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(54) Title: TRIPHENYLMETHANE KINESIN INHIBITORS

(57) Abstract: Triphenylmethane derivatives of the formula (I) are disclosed. The compounds are inhibitors of the mitotic kinesin KSP and are useful in the treatment of cellular proliferative diseases, such as cancer, hyperplasias, restenosis, cardiac hypertrophy, immune disorders and inflammation

TRIPHENYLMETHANE KINESIN INHIBITORS

RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. §119(e) to USSN 60/263,015, which is incorporated by reference in their entirety for all purposes.

FIELD OF THE INVENTION

[0002] This invention relates to triphenylmethane derivatives which are inhibitors of the mitotic kinesin KSP and are useful in the treatment of cellular proliferative diseases, for example cancer, hyperplasias, restenosis, cardiac hypertrophy, immune disorders and inflammation.

BACKGROUND OF THE INVENTION

[0003] Among the therapeutic agents used to treat cancer are the taxanes and vinca alkaloids, which act on microtubules. Microtubules are the primary structural element of the mitotic spindle. The mitotic spindle is responsible for distribution of replicate copies of the genome to each of the two daughter cells that result from cell division. It is presumed that disruption of the mitotic spindle by these drugs results in inhibition of cancer cell division, and induction of cancer cell death. However, microtubules form other types of cellular structures, including tracks for intracellular transport in nerve processes. Because these agents do not specifically target mitotic spindles, they have side effects that limit their usefulness.

[0004] Improvements in the specificity of agents used to treat cancer is of considerable interest because of the therapeutic benefits which would be realized if the side effects associated with the administration of these agents could be reduced. Traditionally, dramatic improvements in the treatment of cancer are associated with identification of therapeutic agents acting through novel mechanisms. Examples of this include not only the taxanes, but also the camptothecin class of topoisomerase I inhibitors. From both of these perspectives, mitotic kinesins are attractive targets for new anti-cancer agents.

[0005] Mitotic kinesins are enzymes essential for assembly and function of the mitotic spindle, but are not generally part of other microtubule structures, such as in nerve processes. Mitotic kinesins play essential roles during all phases of mitosis. These enzymes are "molecular motors" that transform energy released by hydrolysis of ATP

into mechanical force which drives the directional movement of cellular cargoes along microtubules. The catalytic domain sufficient for this task is a compact structure of approximately 340 amino acids. During mitosis, kinesins organize microtubules into the bipolar structure that is the mitotic spindle. Kinesins mediate movement of chromosomes along spindle microtubules, as well as structural changes in the mitotic spindle associated with specific phases of mitosis. Experimental perturbation of mitotic kinesin function causes malformation or dysfunction of the mitotic spindle, frequently resulting in cell cycle arrest and cell death.

[0006] Among the mitotic kinesins which have been identified is KSP. KSP belongs to an evolutionarily conserved kinesin subfamily of plus end-directed microtubule motors that assemble into bipolar homotetramers consisting of antiparallel homodimers. During mitosis KSP associates with microtubules of the mitotic spindle. Microinjection of antibodies directed against KSP into human cells prevents spindle pole separation during prometaphase, giving rise to monopolar spindles and causing mitotic arrest and induction of programmed cell death. KSP and related kinesins in other, non-human, organisms, bundle antiparallel microtubules and slide them relative to one another, thus forcing the two spindle poles apart. KSP may also mediate in anaphase B spindle elongation and focussing of microtubules at the spindle pole.

[0007] Human KSP (also termed HsEg5) has been described (Blangy, et al., Cell, 83:1159-69 (1995); Whitehead, et al., Arthritis Rheum., 39:1635-42 (1996); Galgio et al., J. Cell Biol., 135:339-414 (1996); Blangy, et al., J Biol. Chem., 272:19418-24 (1997); Blangy, et al., Cell Motil Cytoskeleton, 40:174-82 (1998); Whitehead and Rattner, J. Cell Sci., 111:2551-61 (1998); Kaiser, et al., JBC 274:18925-31 (1999); GenBank accession numbers: X85137, NM004523 and U37426) , and a fragment of the KSP gene (TRIP5) has been described (Lee, et al., Mol Endocrinol., 9:243-54 (1995); GenBank accession number L40372). Xenopus KSP homologs (Eg5), as well as Drosophila KLP61 F/KRP1 30 have been reported.

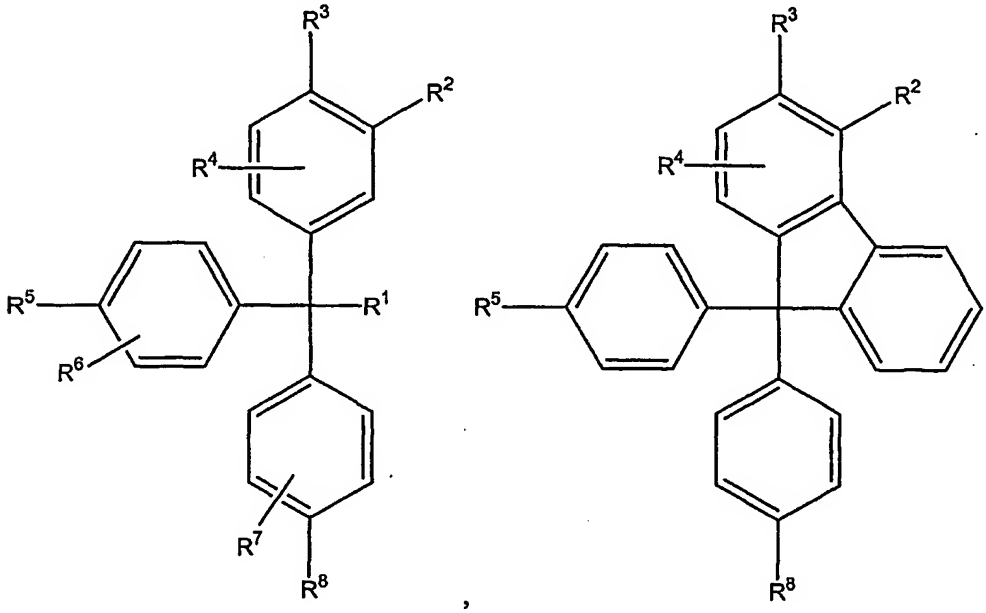
[0008] Mitotic kinesins are attractive targets for the discovery and development of novel mitotic chemotherapeutics. Accordingly, it is an object of the present invention to provide methods and compositions useful in the inhibition of KSP, a mitotic kinesin.

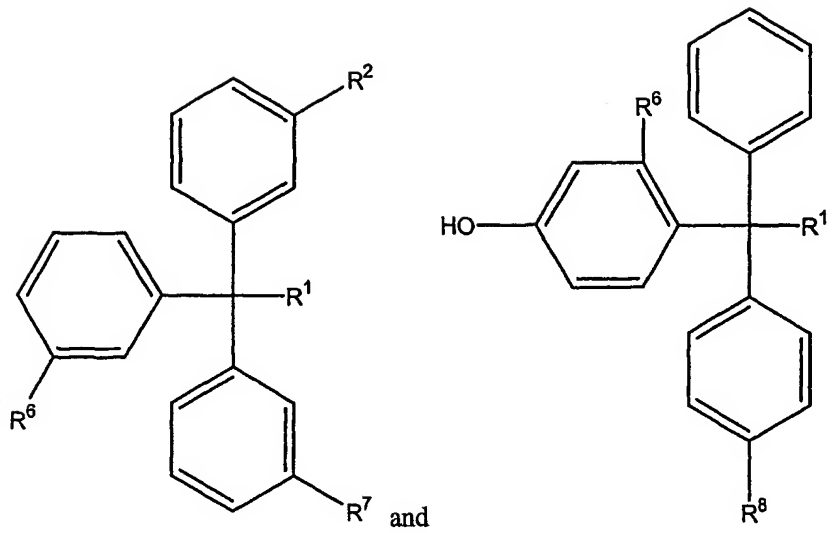
[0009] Certain triphenylmethanes have been disclosed as inhibitors of Ca++ activated potassium channels (WO 97/345780), but inhibition of KSP by triphenylmethanes has not been described.

SUMMARY OF THE INVENTION

[0010] In accordance with the objects outlined above, the present invention provides compositions and methods that can be used to treat diseases of proliferating cells. The compositions are KSP inhibitors, particularly human KSP inhibitors.

[0011] In one aspect, the invention relates to methods for treating cellular proliferative diseases, for treating disorders associated with KSP kinesin activity, and for inhibiting KSP kinesin. The methods employ compounds of the formula:





and

wherein

R^1 is hydrogen or lower alkyl;

R^2 is chosen from H, -OH, -F, $-NH_2$, and $-NO_2$;

R^3 is chosen from H, -COOH, -O-(alkyl), and -OH;

R^4 is chosen from H, -OH, and -COO(alkyl);

R^5 is chosen from -S-(alkyl), $-NH_2$, $-N(alkyl)_2$, -OH, -O-(alkyl) and SO_2CH_3;

R^6 is chosen from H, $-N(alkyl)_2$, -OH and -COOH;

R^7 is chosen from H, $-N(alkyl)_2$, -OH and -COOH; and

R^8 is chosen from -S-(alkyl), $-NH_2$, $-N(alkyl)_2$, -OH, -O-(alkyl) and SO_2CH_3, wherein at least one of R^2, R^3 and R^4 must be other than hydrogen.

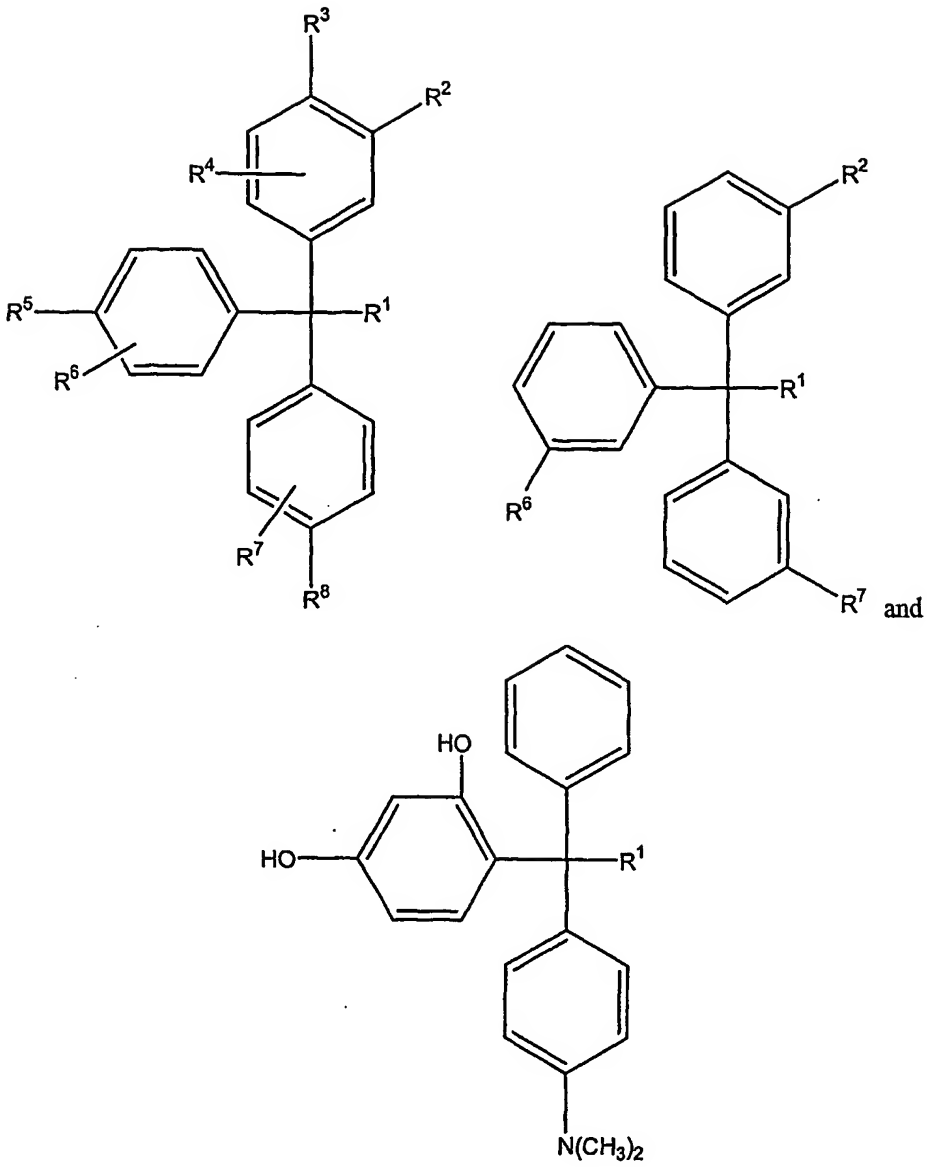
[0012] Diseases and disorders that respond to therapy with compounds of the invention include cancer, hyperplasia, restenosis, cardiac hypertrophy, immune disorders and inflammation.

[0013] In another aspect, the invention relates to compounds useful in inhibiting KSP kinesin. The compounds have the structures shown above.

[0014] In an additional aspect, the present invention provides methods of screening for compounds that will bind to a KSP kinesin, for example compounds that will displace or compete with the binding of the compositions of the invention. The methods comprise combining a labeled compound of the invention, a KSP kinesin, and at least one candidate agent and determining the binding of the candidate bioactive agent to the KSP kinesin.

[0015] In a further aspect, the invention provides methods of screening for modulators of KSP kinesin activity. The methods comprise combining a composition of the invention, a KSP kinesin, and at least one candidate agent and determining the effect of the candidate bioactive agent on the KSP kinesin activity.

[0016] In another aspect, the invention relates to novel compounds that show activity in inhibiting KSP kinesin. The compounds have the structures:



wherein the substituents are as defined before.

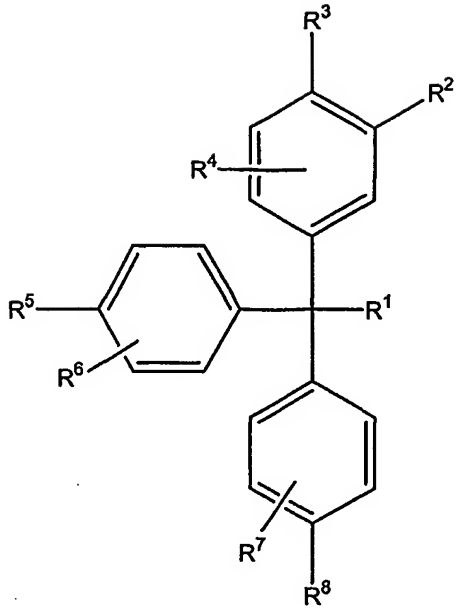
DETAILED DESCRIPTION OF THE INVENTION

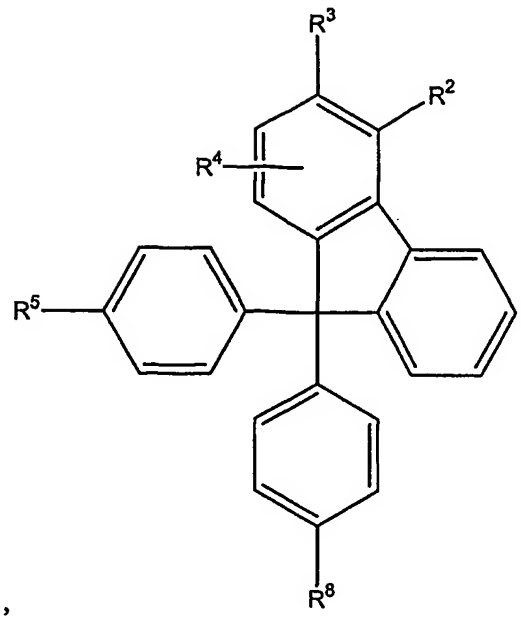
[0017] The present invention is directed to a class of novel triphenylmethanes that are modulators of mitotic kinesins. By inhibiting or modulating mitotic kinesins, but not other kinesins (e.g., transport kinesins), specific inhibition of cellular proliferation is accomplished. Thus, the present invention capitalizes on the finding that perturbation of

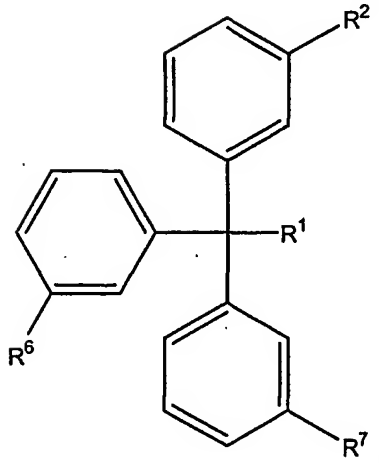
mitotic kinesin function causes malformation or dysfunction of mitotic spindles, frequently resulting in cell cycle arrest and cell death. The methods of inhibiting a human KSP kinesin comprise contacting an inhibitor of the invention with a KSP kinesin, particularly human KSP kinesins, including fragments and variants of KSP. The inhibition can be of the ATP hydrolysis activity of the KSP kinesin and/or the mitotic spindle formation activity, such that the mitotic spindles are disrupted. Meiotic spindles may also be disrupted.

[0018] An object of the present invention is to develop inhibitors and modulators of mitotic kinesins, in particular KSP, for the treatment of disorders associated with cell proliferation. Traditionally, dramatic improvements in the treatment of cancer, one type of cell proliferative disorder, have been associated with identification of therapeutic agents acting through novel mechanisms. Examples of this include not only the taxane class of agents that appear to act on microtubule formation, but also the camptothecin class of topoisomerase I inhibitors. The compositions and methods described herein can differ in their selectivity and are preferably used to treat diseases of proliferating cells, including, but not limited to cancer, hyperplasias, restenosis, cardiac hypertrophy, immune disorders and inflammation.

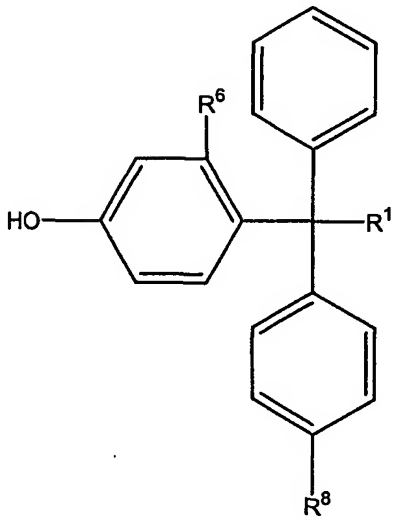
[0019] Accordingly, the present invention relates to methods employing triphenylmethanes of the formula:





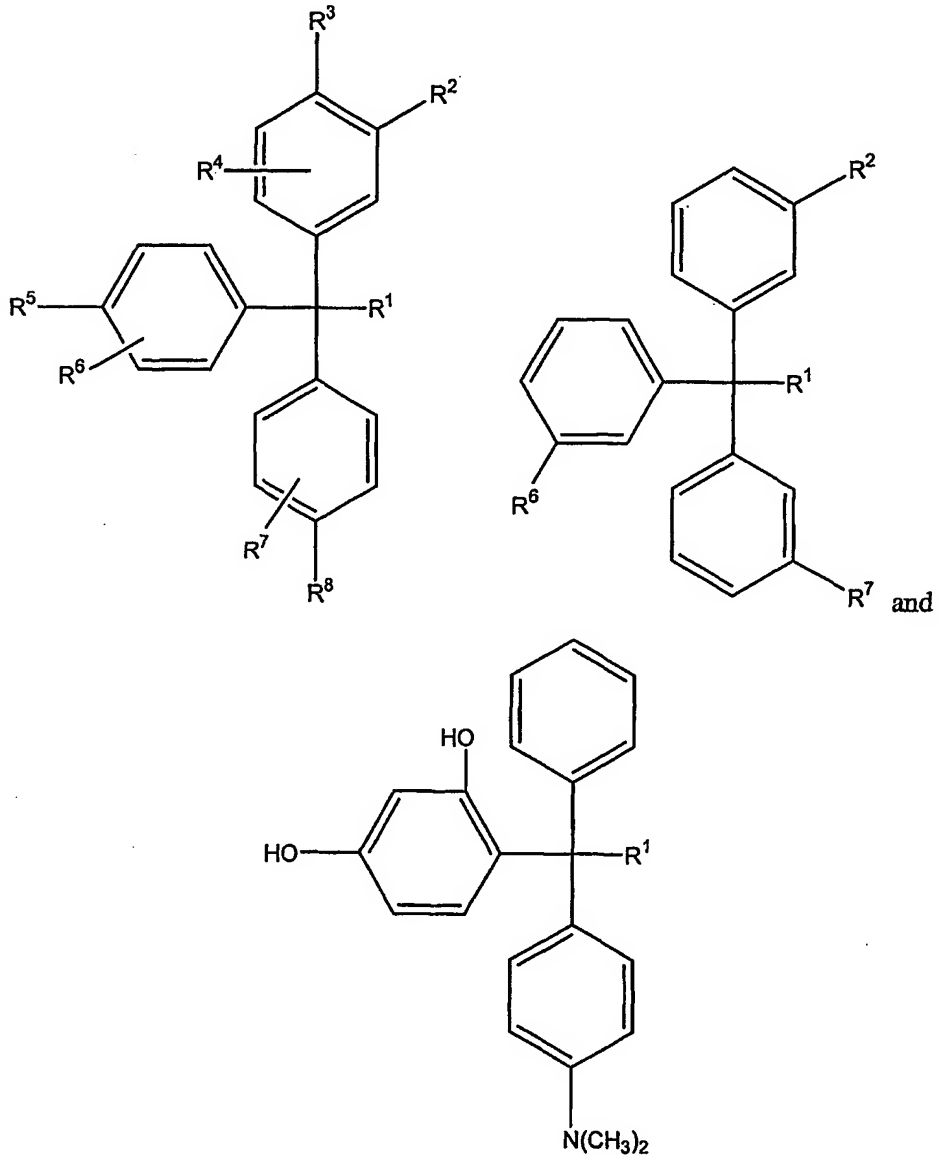


and



[0020] All of the compounds falling within the foregoing parent genus and its subgenera are useful as kinesin inhibitors, but not all the compounds are novel. In particular, certain known species fall within the genus, although no utility in inhibiting kinesin has been suggested for these species. Any narrowing of the claims or specific exceptions that might be added to these claims reflect applicants' intent to avoid claiming

subject matter that, while functionally part of the inventive concept, is not patentable to them for reasons having nothing to do with the scope of their invention. In particular, the novel compounds that are the subject of the claims are described by the formulas:



and

when R^2 is -OH and R^3 and R^4 are hydrogen, R^5 and R^8 cannot be $-N(CH_3)_2$.

[0021] Preferred compounds of the methods and compositions are those in which: (1) R^2 is chosen from -OH, -F, $-NH_2$, and $-NO_2$; (2) R^2 is H and R^3 is -OH, -

COOH or -OCH_3; (3) R^5 and R^8 are chosen from -N(alkyl)$_2$ and -OH; (4) R^2 is chosen from -OH, -F, and -NH_2; (5) R^6 and R^7 are hydrogen or R^6 and R^7 are -N(alkyl)$_2$; and R^5 and R^8 are chosen from -S-CH_3, -N(lower-alkyl)$_2$ and SO_2CH_3.

Definitions

[0022] Alkyl is intended to include linear, branched, or cyclic hydrocarbon structures and combinations thereof having 12 or fewer carbons. Lower alkyl refers to alkyl groups of from 1 to 5 carbon atoms. Examples of lower alkyl groups include methyl, ethyl, propyl, isopropyl, butyl, s-and t-butyl and the like. Cycloalkyl is a subset of alkyl and includes cyclic hydrocarbon groups of from 3 to 12 carbon atoms. Examples of cycloalkyl groups include c-propyl, c-butyl, c-pentyl, norbornyl, adamantyl and the like. When an alkyl residue having a specific number of carbons is named, all geometric isomers having that number of carbons are intended to be encompassed; thus, for example, "butyl" is meant to include n-butyl, sec-butyl, isobutyl and t-butyl; "propyl" includes n-propyl and isopropyl.

[0023] Alkoxy or alkoxyl refers to groups of from 1 to 8 carbon atoms of a straight, branched, cyclic configuration and combinations thereof attached to the parent structure through an oxygen. Examples include methoxy, ethoxy, propoxy, isopropoxy, cyclopropyloxy, cyclohexyloxy and the like. Lower-alkoxy refers to groups containing one to four carbons, and such are preferred.

[0024] Halogen refers to fluorine, chlorine, bromine or iodine. Fluorine, chlorine and bromine are preferred.

[0025] Some of the compounds described herein contain one or more asymmetric centers (e.g. the methine carbon when each of the phenyl rings is differently substituted) and may thus give rise to enantiomers, diastereomers (e.g. when R^1 contains a stereogenic center), and other stereoisomeric forms that may be defined, in terms of absolute stereochemistry, as (R)- or (S)-. The present invention is meant to include all such possible isomers, including racemic mixtures, optically pure forms and intermediate mixtures. Optically active (R)- and (S)- isomers may be prepared using chiral synthons or chiral reagents, or resolved using conventional techniques. When the compounds described herein contain olefinic double bonds or other centers of geometric asymmetry,

and unless specified otherwise, it is intended that the compounds include both E and Z geometric isomers. Likewise, all tautomeric forms are also intended to be included.

[0026] When desired, the R- and S-isomers may be resolved by methods known to those skilled in the art, for example by formation of diastereoisomeric salts or complexes which may be separated, for example, by crystallisation; via formation of diastereoisomeric derivatives which may be separated, for example, by crystallisation, gas-liquid or liquid chromatography; selective reaction of one enantiomer with an enantiomer-specific reagent, for example enzymatic oxidation or reduction, followed by separation of the modified and unmodified enantiomers; or gas-liquid or liquid chromatography in a chiral environment, for example on a chiral support, such as silica with a bound chiral ligand or in the presence of a chiral solvent. It will be appreciated that where the desired enantiomer is converted into another chemical entity by one of the separation procedures described above, a further step may be required to liberate the desired enantiomeric form. Alternatively, specific enantiomer may be synthesized by asymmetric synthesis using optically active reagents, substrates, catalysts or solvents, or by converting one enantiomer to the other by asymmetric transformation.

[0027] The compositions of the invention are synthesized as outlined below, utilizing techniques well known in the art. Once made, the compositions of the invention find use in a variety of applications. As will be appreciated by those in the art, mitosis may be altered in a variety of ways; that is, one can affect mitosis either by increasing or decreasing the activity of a component in the mitotic pathway. Stated differently, mitosis may be affected (e.g., disrupted) by disturbing equilibrium, either by inhibiting or activating certain components. Similar approaches may be used to alter meiosis.

[0028] In a preferred embodiment, the compositions of the invention are used to modulate mitotic spindle formation, thus causing prolonged cell cycle arrest in mitosis. By "modulate" herein is meant altering mitotic spindle formation, including increasing and decreasing spindle formation. By "mitotic spindle formation" herein is meant organization of microtubules into bipolar structures by mitotic kinesins. By "mitotic spindle dysfunction" herein is meant mitotic arrest and monopolar spindle formation.

[0029] The compositions of the invention are useful to bind to and/or modulate the activity of a mitotic kinesin, KSP. In a preferred embodiment, the KSP is human KSP, although KSP kinesins from other organisms may also be used. In this context,

modulate means either increasing or decreasing spindle pole separation, causing malformation, i.e., splaying, of mitotic spindle poles, or otherwise causing morphological perturbation of the mitotic spindle. Also included within the definition of KSP for these purposes are variants and/or fragments of KSP. See U.S. Patent Application "Methods of Screening for Modulators of Cell Proliferation and Methods of Diagnosing Cell Proliferation States", filed Oct. 27, 1999 (U.S. Serial Number 09/428,156), hereby incorporated by reference in its entirety. In addition, other mitotic kinesins may be used in the present invention. However, the compositions of the invention have been shown to have specificity for KSP.

[0030] For assay of activity, generally either KSP or a compound according to the invention is non-diffusably bound to an insoluble support having isolated sample receiving areas (e.g., a microtiter plate, an array, etc.). The insoluble support may be made of any composition to which the compositions can be bound, is readily separated from soluble material, and is otherwise compatible with the overall method of screening. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports include microtiter plates, arrays, membranes and beads. These are typically made of glass, plastic (e.g., polystyrene), polysaccharides, nylon or nitrocellulose, Teflon™, etc. Microtiter plates and arrays are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples. The particular manner of binding of the composition is not crucial so long as it is compatible with the reagents and overall methods of the invention, maintains the activity of the composition and is nondiffusable. Preferred methods of binding include the use of antibodies (which do not sterically block either the ligand binding site or activation sequence when the protein is bound to the support), direct binding to "sticky" or ionic supports, chemical crosslinking, the synthesis of the protein or agent on the surface, etc. Following binding of the protein or agent, excess unbound material is removed by washing. The sample receiving areas may then be blocked through incubation with bovine serum albumin (BSA), casein or other innocuous protein or other moiety.

[0031] The antimitotic agents of the invention may be used on their own to modulate the activity of a mitotic kinesin, particularly KSP. In this embodiment, the mitotic agents of the invention are combined with KSP and the activity of KSP is

assayed. Kinesin activity is known in the art and includes one or more kinesin activities. Kinesin activities include the ability to affect ATP hydrolysis; microtubule binding; gliding and polymerization/depolymerization (effects on microtubule dynamics); binding to other proteins of the spindle; binding to proteins involved in cell-cycle control; serving as a substrate to other enzymes; such as kinases or proteases; and specific kinesin cellular activities such as spindle pole separation.

[0032] Methods of performing motility assays are well known to those of skill in the art. (See e.g., Hall, et al. (1996), Biophys. J., 71: 3467-3476, Turner et al., 1996, Anal. Biochem. 242 (1):20-5; Gittes et al., 1996, Biophys. J. 70(l): 418-29; Shirakawa et al., 1995, J. Exp. BioL 198: 1809-15; Winkelmann et al., 1995, Biophys. J. 68: 2444-53; Winkelmann et al., 1995, Biophys. J. 68: 72S.)

[0033] Methods known in the art for determining ATPase hydrolysis activity also can be used. Preferably, solution based assays are utilized. U.S. application 09/314,464, filed May 18, 1999, hereby incorporated by reference in its entirety, describes such assays. Alternatively, conventional methods are used. For example, P_i release from kinesin can be quantified. In one preferred embodiment, the ATPase hydrolysis activity assay utilizes 0.3 M PCA (perchloric acid) and malachite green reagent (8.27 mM sodium molybdate II, 0.33 mM malachite green oxalate, and 0.8 mM Triton X-1 00). To perform the assay, 10 μL of reaction is quenched in 90 μL of cold 0.3 M PCA. Phosphate standards are used so data can be converted to mM inorganic phosphate released. When all reactions and standards have been quenched in PCA, 100 μL of malachite green reagent is added to the relevant wells in e.g., a microtiter plate. The mixture is developed for 10-15 minutes and the plate is read at an absorbance of 650 nm. If phosphate standards were used, absorbance readings can be converted to mM P_i and plotted over time. Additionally, ATPase assays known in the art include the luciferase assay.

[0034] ATPase activity of kinesin motor domains also can be used to monitor the effects of modulating agents. In one embodiment ATPase assays of kinesin are performed in the absence of microtubules. In another embodiment, the ATPase assays are performed in the presence of microtubules. Different types of modulating agents can be detected in the above assays. In a preferred embodiment, the effect of a modulating agent is independent of the concentration of microtubules and ATP. In another

embodiment, the effect of the agents on kinesin ATPase can be decreased by increasing the concentrations of ATP, microtubules or both. In yet another embodiment, the effect of the modulating agent is increased by increasing concentrations of ATP, microtubules or both.

[0035] Agents that modulate the biochemical activity of KSP in vitro may then be screened in vivo. Methods for such agents in vivo include assays of cell cycle distribution, cell viability, or the presence, morphology, activity, distribution, or amount of mitotic spindles. Methods for monitoring cell cycle distribution of a cell population, for example, by flow cytometry, are well known to those skilled in the art, as are methods for determining cell viability. See for example, U.S. Patent Application "Methods of Screening for Modulators of Cell Proliferation and Methods of Diagnosing Cell Proliferation States," filed Oct. 22, 1999, serial number 09/428,156, hereby incorporated by reference in its entirety.

[0036] In addition to the assays described above, microscopic methods for monitoring spindle formation and malformation are well known to those of skill in the art (see, e.g., Whitehead and Rattner (1998), J. Cell Sci. 111:2551-61; Galgio et al, (1996) J. Cell biol., 135:399-414).

[0037] The compositions of the invention inhibit the KSP kinesin. One measure of inhibition is IC_{50}, defined as the concentration of the composition at which the activity of KSP is decreased by fifty percent. Preferred compositions have IC_{50}'s of less than about 1 mM, with preferred embodiments having IC_{50}'s of less than about 100 μM, with more preferred embodiments having IC_{50}'s of less than about 10 μM, with particularly preferred embodiments having IC_{50}'s of less than about 1 μM, and especially preferred embodiments having IC_{50}'s of less than about 500 nM. Measurement of IC_{50} is done using an ATPase assay.

[0038] Another measure of inhibition is K_i. For compounds with IC_{50}'s less than 1 μM, the K_i or K_d is defined as the dissociation rate constant for the interaction of the triphenylmethane with KSP. Preferred compounds have K_i's of less than about 100 μM, with preferred embodiments having K_i's of less than about 10 μM, and particularly preferred embodiments having K_i's of less than about 1 μM and especially preferred embodiments having K_i's of less than about 500 nM.

[0039] Another measure of inhibition is GI_{50}, defined as the concentration of the compound that results in a decrease in the rate of cell growth by fifty percent. Preferred compounds have GI_{50}'s of less than about 1 mM. The level of preferability of embodiments is a function of their GI_{50} : those having GI_{50}'s of less than about 20 μM are more preferred; those having GI_{50}'s of 10 μM more so; those having GI_{50} of less than about 1 μM more so; those having GI_{50}'s of 500 nM more so. Measurement of GI_{50} is done using a cell proliferation assay.

[0040] The compositions of the invention are used to treat cellular proliferation diseases. Disease states which can be treated by the methods and compositions provided herein include, but are not limited to, cancer (further discussed below), autoimmune disease, arthritis, graft rejection, inflammatory bowel disease, proliferation induced after medical procedures, including, but not limited to, surgery, angioplasty, and the like. It is appreciated that in some cases the cells may not be in a hyper or hypo proliferation state (abnormal state) and still require treatment. For example, during wound healing, the cells may be proliferating "normally", but proliferation enhancement may be desired. Similarly, as discussed above, in the agriculture arena, cells may be in a "normal" state, but proliferation modulation may be desired to enhance a crop by directly enhancing growth of a crop, or by inhibiting the growth of a plant or organism which adversely affects the crop. Thus, in one embodiment, the invention herein includes application to cells or individuals afflicted or impending affliction with any one of these disorders or states.

[0041] The compositions and methods provided herein are particularly deemed useful for the treatment of cancer including solid tumors such as skin, breast, brain, cervical carcinomas, testicular carcinomas, etc. More particularly, cancers that may be treated by the compositions and methods of the invention include, but are not limited to: Cardiac: sarcoma (angiosarcoma, fibrosarcoma, rhabdomyosarcoma, liposarcoma), myxoma, rhabdomyoma, fibroma, lipoma and teratoma; Lung: bronchogenic carcinoma (squamous cell, undifferentiated small cell, undifferentiated large cell, adenocarcinoma), alveolar (bronchiolar) carcinoma, bronchial adenoma, sarcoma, lymphoma, chondromatous hamartoma, mesothelioma; Gastrointestinal: esophagus (squamous cell carcinoma, adenocarcinoma, leiomyosarcoma, lymphoma), stomach (carcinoma, lymphoma, leiomyosarcoma), pancreas (ductal adenocarcinoma, insulinoma,

glucagonoma, gastrinoma, carcinoid tumors, vipoma), small bowel (adenocarcinoma, lymphoma, carcinoid tumors, Karposi's sarcoma, leiomyoma, hemangioma, lipoma, neurofibroma, fibroma), large bowel (adenocarcinoma, tubular adenoma, villous adenoma, hamartoma, leiomyoma); Genitourinary tract: kidney (adenocarcinoma, Wilm's tumor (nephroblastoma), lymphoma, leukemia), bladder and urethra (squamous cell carcinoma, transitional cell carcinoma, adenocarcinoma), prostate (adenocarcinoma, sarcoma), testis (seminoma, teratoma, embryonal carcinoma, teratocarcinoma, choriocarcinoma, sarcoma, interstitial cell carcinoma, fibroma, fibroadenoma, adenomatoid tumors, lipoma); Liver: hepatoma (hepatocellular carcinoma), cholangiocarcinoma, hepatoblastoma, angiosarcoma, hepatocellular adenoma, hemangioma; Bone: osteogenic sarcoma (osteosarcoma), fibrosarcoma, malignant fibrous histiocytoma, chondrosarcoma, Ewing's sarcoma, malignant lymphoma (reticulum cell sarcoma), multiple myeloma, malignant giant cell tumor chordoma, osteochronfroma (osteocartilaginous exostoses), benign chondroma, chondroblastoma, chondromyxofibroma, osteoid osteoma and giant cell tumors; Nervous system: skull (osteoma, hemangioma, granuloma, xanthoma, osteitis deformans), meninges (meningioma, meningiosarcoma, gliomatosis), brain (astrocytoma, medulloblastoma, glioma, ependymoma, germinoma (pinealoma), glioblastoma multiform, oligodendroglioma, schwannoma, retinoblastoma, congenital tumors), spinal cord neurofibroma, meningioma, glioma, sarcoma); Gynecological: uterus (endometrial carcinoma), cervix (cervical carcinoma, pre-tumor cervical dysplasia), ovaries (ovarian carcinoma (serous cystadenocarcinoma, mucinous cystadenocarcinoma, unclassified carcinoma), granulosa-thecal cell tumors, Sertoli-Leydig cell tumors, dysgerminoma, malignant teratoma), vulva (squamous cell carcinoma, intraepithelial carcinoma, adenocarcinoma, fibrosarcoma, melanoma), vagina (clear cell carcinoma, squamous cell carcinoma, botryoid sarcoma (embryonal rhabdomyosarcoma), fallopian tubes (carcinoma); Hematologic: blood (myeloid leukemia (acute and chronic), acute lymphoblastic leukemia, chronic lymphocytic leukemia, myeloproliferative diseases, multiple myeloma, myelodysplastic syndrome), Hodgkin's disease, non-Hodgkin's lymphoma (malignant lymphoma); Skin: malignant melanoma, basal cell carcinoma, squamous cell carcinoma, Karposi's sarcoma, moles dysplastic nevi, lipoma, angioma, dermatofibroma, keloids, psoriasis; and Adrenal glands: neuroblastoma. Thus, the term

"cancerous cell" as provided herein, includes a cell afflicted by any one of the above identified conditions.

[0042] Accordingly, the compositions of the invention are administered to cells. By "administered" herein is meant administration of a therapeutically effective dose of the mitotic agents of the invention to a cell either in cell culture or in a patient. By "therapeutically effective dose" herein is meant a dose that produces the effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques. As is known in the art, adjustments for systemic versus localized delivery, age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the condition may be necessary, and will be ascertainable with routine experimentation by those skilled in the art. By "cells" herein is meant cells in which mitosis or meiosis can be altered.

[0043] A "patient" for the purposes of the present invention includes both humans and other animals, particularly mammals, and other organisms. Thus the methods are applicable to both human therapy and veterinary applications. In the preferred embodiment the patient is a mammal, and in the most preferred embodiment the patient is human.

[0044] Mitotic agents having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a patient, as described herein. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways as discussed below. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%. The agents may be administered alone or in combination with other treatments, i.e., radiation, or other chemotherapeutic agents.

[0045] In a preferred embodiment, the pharmaceutical compositions are in a water soluble form, such as pharmaceutically acceptable salts, which is meant to include both acid and base addition salts. "Pharmaceutically acceptable acid addition salt" refers to those salts that retain the biological effectiveness of the free bases and that are not biologically or otherwise undesirable, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid,

maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like. "Pharmaceutically acceptable base addition salts" include those derived from inorganic bases such as sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum salts and the like. Particularly preferred are the ammonium, potassium, sodium, calcium, and magnesium salts. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, and ethanolamine.

[0046] The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents. The pharmaceutical compositions may also include one or more of the following: carrier proteins such as serum albumin; buffers; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents; sweeteners and other flavoring agents; coloring agents; and polyethylene glycol. Additives are well known in the art, and are used in a variety of formulations.

[0047] The administration of the mitotic agents of the present invention can be done in a variety of ways as discussed above, including, but not limited to, orally, subcutaneously, intravenously, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, or intraocularly. In some instances, for example, in the treatment of wounds and inflammation, the anti-mitotic agents may be directly applied as a solution or spray.

[0048] To employ the compounds of the invention in a method of screening for compounds that bind to KSP kinesin, the KSP is bound to a support, and a compound of the invention (which is a mitotic agent) is added to the assay. Alternatively, the

compound of the invention is bound to the support and KSP is added. Classes of compounds among which novel binding agents may be sought include specific antibodies, non-natural binding agents identified in screens of chemical libraries, peptide analogs, etc. Of particular interest are screening assays for candidate agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including labeled in vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, functional assays (phosphorylation assays, etc.) and the like.

[0049] The determination of the binding of the mitotic agent to KSP may be done in a number of ways. In a preferred embodiment, the mitotic agent (the compound of the invention) is labeled, for example, with a fluorescent or radioactive moiety and binding determined directly. For example, this may be done by attaching all or a portion of KSP to a solid support, adding a labeled mitotic agent (for example a compound of the invention in which at least one atom has been replaced by a detectable isotope), washing off excess reagent, and determining whether the amount of the label is that present on the solid support. Various blocking and washing steps may be utilized as is known in the art.

[0050] By "labeled" herein is meant that the compound is either directly or indirectly labeled with a label which provides a detectable signal, e.g., radioisotope, fluorescent tag, enzyme, antibodies, particles such as magnetic particles, chemiluminescent tag, or specific binding molecules, etc. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule which provides for detection, in accordance with known procedures, as outlined above. The label can directly or indirectly provide a detectable signal.

[0051] In some embodiments, only one of the components is labeled. For example, the kinesin proteins may be labeled at tyrosine positions using ^{125}I, or with fluorophores. Alternatively, more than one component may be labeled with different labels; using ^{125}I for the proteins, for example, and a fluorophor for the mitotic agents.

[0052] The compounds of the invention may also be used as competitors to screen for additional drug candidates. "Candidate bioactive agent" or "drug candidate" or grammatical equivalents as used herein describe any molecule, e.g., protein,

oligopeptide, small organic molecule, polysaccharide, polynucleotide, etc., to be tested for bioactivity. They may be capable of directly or indirectly altering the cellular proliferation phenotype or the expression of a cellular proliferation sequence, including both nucleic acid sequences and protein sequences. In other cases, alteration of cellular proliferation protein binding and/or activity is screened. Screens of this sort may be performed either in the presence or absence of microtubules. In the case where protein binding or activity is screened, preferred embodiments exclude molecules already known to bind to that particular protein, for example, polymer structures such as microtubules, and energy sources such as ATP. Preferred embodiments of assays herein include candidate agents which do not bind the cellular proliferation protein in its endogenous native state termed herein as "exogenous" agents. In another preferred embodiment, exogenous agents further exclude antibodies to KSP.

[0053] Candidate agents can encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding and lipophilic binding, and typically include at least an amine, carbonyl, hydroxyl, ether, or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are peptides.

[0054] Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or

random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

[0055] Competitive screening assays may be done by combining KSP and a drug candidate in a first sample. A second sample comprises a mitotic agent, KSP and a drug candidate. This may be performed in either the presence or absence of microtubules. The binding of the drug candidate is determined for both samples, and a change, or difference in binding between the two samples indicates the presence of an agent capable of binding to KSP and potentially modulating its activity. That is, if the binding of the drug candidate is different in the second sample relative to the first sample, the drug candidate is capable of binding to KSP.

[0056] In a preferred embodiment, the binding of the candidate agent is determined through the use of competitive binding assays. In this embodiment, the competitor is a binding moiety known to bind to KSP, such as an antibody, peptide, binding partner, ligand, etc. Under certain circumstances, there may be competitive binding as between the candidate agent and the binding moiety, with the binding moiety displacing the candidate agent.

[0057] In one embodiment, the candidate agent is labeled. Either the candidate agent, or the competitor, or both, is added first to KSP for a time sufficient to allow binding, if present. Incubations may be performed at any temperature which facilitates optimal activity, typically between 4 and 40°C.

[0058] Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high throughput screening. Typically between 0.1 and 1 hour will be sufficient. Excess reagent is generally removed or washed away. The second component is then added, and the presence or absence of the labeled component is followed, to indicate binding.

[0059] In a preferred embodiment, the competitor is added first, followed by the candidate agent. Displacement of the competitor is an indication the candidate agent is binding to KSP and thus is capable of binding to, and potentially modulating, the activity of KSP. In this embodiment, either component can be labeled. Thus, for example, if the competitor is labeled, the presence of label in the wash solution indicates displacement by the agent. Alternatively, if the candidate agent is labeled, the presence of the label on the support indicates displacement.

[0060] In an alternative embodiment, the candidate agent is added first, with incubation and washing, followed by the competitor. The absence of binding by the competitor may indicate the candidate agent is bound to KSP with a higher affinity. Thus, if the candidate agent is labeled, the presence of the label on the support, coupled with a lack of competitor binding, may indicate the candidate agent is capable of binding to KSP.

[0061] It may be of value to identify the binding site of KSP. This can be done in a variety of ways. In one embodiment, once KSP has been identified as binding to the mitotic agent, KSP is fragmented or modified and the assays repeated to identify the necessary components for binding.

[0062] Modulation is tested by screening for candidate agents capable of modulating the activity of KSP comprising the steps of combining a candidate agent with KSP, as above, and determining an alteration in the biological activity of KSP. Thus, in this embodiment, the candidate agent should both bind to KSP (although this may not be necessary), and alter its biological or biochemical activity as defined herein. The methods include both in vitro screening methods and in vivo screening of cells for alterations in cell cycle distribution, cell viability, or for the presence, morpohology, activity, distribution, or amount of mitotic spindles, as are generally outlined above.

[0063] Alternatively, differential screening may be used to identify drug candidates that bind to the native KSP, but cannot bind to modified KSP.

[0064] Positive controls and negative controls may be used in the assays. Preferably all control and test samples are performed in at least triplicate to obtain statistically significant results. Incubation of all samples is for a time sufficient for the binding of the agent to the protein. Following incubation, all samples are washed free of non-specifically bound material and the amount of bound, generally labeled agent determined. For example, where a radiolabel is employed, the samples may be counted in a scintillation counter to determine the amount of bound compound.

[0065] A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g., albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be

used. The mixture of components may be added in any order that provides for the requisite binding.

[0066] The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference in their entirety.

EXAMPLES

Abbreviations and Definitions

[0067] The following abbreviations and terms have the indicated meanings throughout:

Ac	=	acetyl
BNB	=	4-bromomethyl-3-nitrobenzoic acid
Boc	=	t-butyloxy carbonyl
Bu	=	butyl
c-	=	cyclo
CBZ	=	carbobenzoxy = benzyloxycarbonyl
DBU	=	diazabicyclo[5.4.0]undec-7-ene
DCM	=	dichloromethane = methylene chloride = CH_2Cl_2
DCE	=	dichloroethane
DEAD	=	diethyl azodicarboxylate
DIC	=	diisopropylcarbodiimide
DIEA	=	N,N-diisopropylethylamine
DMAP	=	4-N,N-dimethylaminopyridine
DMF	=	N,N-dimethylformamide
DMSO	=	dimethyl sulfoxide
DVB	=	1,4-divinylbenzene
EDCI	=	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EEDQ	=	2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline

Et = ethyl

Fmoc = 9-fluorenylmethoxycarbonyl

GC = gas chromatography

HATU = O-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HMDS = hexamethyldisilazane

HOAc = acetic acid

HOBt = hydroxybenzotriazole

Me = methyl

mesyl = methanesulfonyl

MOM = methoxymethyl

MTBE = methyl t-butyl ether

PEG = polyethylene glycol

Ph = phenyl

PhOH = phenol

PfP = pentafluorophenol

PPTS = pyridinium p-toluenesulfonate

Py = pyridine

PyBroP = bromo-tris-pyrrolidino-phosphonium hexafluorophosphate

rt = room temperature

sat=d = saturated

s- = secondary

t- = tertiary

TBDMS = t-butyldimethylsilyl

TES = triethylsilane

TFA = trifluoroacetic acid

THF = tetrahydrofuran

TMOF = trimethyl orthoformate

TMS = trimethylsilyl

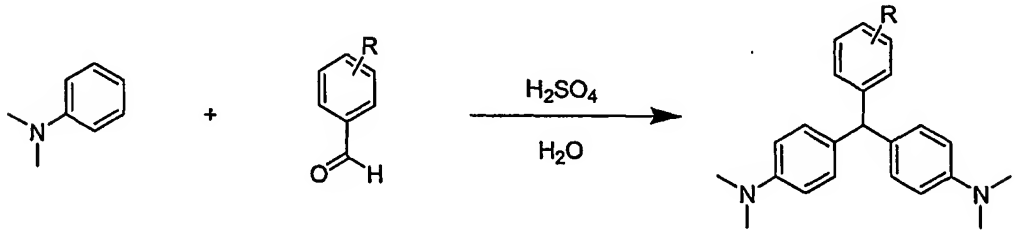
tosyl = p-toluenesulfonyl

Trt = triphenylmethyl

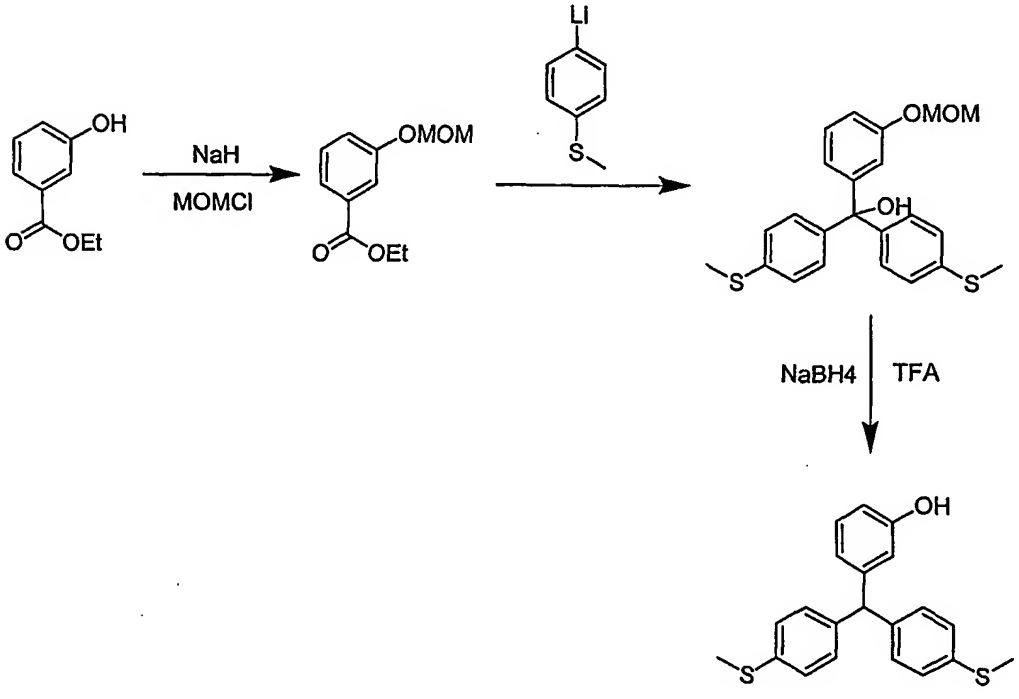
Synthesis of Compounds

[0068] Two general synthetic approaches to the preparation of prototypical triphenylmethanes are shown below. Other triphenylmethanes are made in analogous fashion:

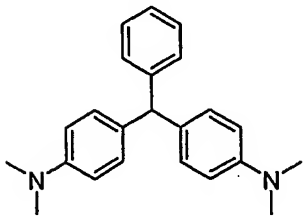
Triphenylmethane Synthesis - Procedure A



Triphenylmethane Synthesis - Procedure B

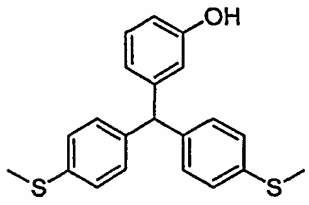


Triphenylmethane Procedure A:



[0069] Dimethylaniline (4 mmol) was combined with benzaldehyde (2 mmol) in water (2 mL) and sulfuric acid (100 L) and heated at 95° C for 48 h. The cooled mixture was washed with DCM (2 x 4ml), neutralized with Na_2CO_3, extracted with DCM (2 x 4 mLl), dried ($MgSO_4$) and evaporated affording product (about 1.50 mmol, 75%)

Triphenylmethane Procedure B:



[0070] Ethyl-3-hydroxy benzoate (7.50 g, 45.2 mmol) was dissolved in anhydrous THF (50 ml) and cooled to 0° C. A 60 % aqueous solution of sodium hydride (1.81g, 45.18 mmol) was added in small portions and the mixture was stirred for 10 min. Chloromethylmethyl ether (4.29 ml, 56.5 mmol) was added over 5 min and the mixture was allowed to warm to RT over 1h. The mixture was combined with DCM(150mL) and washed with water (3x 150mL), dried (MgSO4) and evaporated affording a clear oil (9.62g, 101%)

[0071] 4-Bromothioanisole (812 mg, 4.00 mmol) was dissolved in THF (4 mL) and cooled to –78 °C. 1.6M n-butyl lithium (2.50 mL, 4.00 mmol) was added and the mixture was stirred at –78 °C for 30 min. In a second flask, ethylmethoxymethoxy

benzoate (410 mg, 2.00 mmol) was dissolved in THF (4 mL), and cooled to –78 °C. The first mixture was cannulated into the second and allowed to warm to RT over 1 h. The mixture was dissolved in DCM(10 mL) and washed with saturated ammonium chloride and water, dried ($MgSO_4$) and concentrated.

[0072] To stirring TFA (5 mL) at 0° C was added sodium borohydride crystals(~150 mg). After the reaction slowed, a solution of the material from the previous reaction(150 mg, 0.364 mmol) in DCM (2 mL) was added dropwise over 15 min. 2 drops were added at a time until the color faded to white. Sodium borohydride was added periodically to maintain an excess. After complete addition, the mixture was stirred at 0° C for 15 min. The mixture was dissolved in DCM (25 ml), washed with water (3 x 25 mL), dried ($MgSO_4$) and concentrated. Preparative TLC eluting with 15% ethyl acetate/hexane afforded a thick syrup (60 mg, 46%).

Induction of Mitotic Arrest in Cell Populations Treated with a Triphenylmethane KSP Inhibitor

[0073] FACS analysis to determine cell cycle stage by measuring DNA content was performed as follows. Skov-3 cells (human ovarian cancer) were split 1:10 for plating in 10cm dishes and grown to subconfluence with RPMI 1640 medium containing 5% fetal bovine serum (FBS). The cells were then treated with either 10nM paclitaxel, the test compound or 0.25% DMSO (vehicle for compounds) for 24 hours. Cells were then rinsed off the plates with PBS containing 5mM EDTA, pelleted, washed once in PBS containing 1% FCS, and then fixed overnight in 85% ethanol at 4°C. Before analysis, the cells were pelleted, washed once, and stained in a solution of 10μg propidium iodide and 250μg of ribonuclease (RNAse) A per milliliter at 37°C for half an hour. Flow cytometry analysis was performed on a Becton-Dickinson FACScan, and data from 10,000 cells per sample was analyzed with Modfit software.

[0074] The triphenylmethane compounds, as well as the known anti-mitotic agent paclitaxel, caused a shift in the population of cells from a G0/G1 cell cycle stage (2n DNA content) to a G2/M cell cycle stage (4n DNA content). Other compounds of this class were found to have similar effects.

Monopolar Spindle Formation following Application of a Triphenylmethane KSP Inhibitor

[0075] To determine the nature of the G2/M accumulation, human tumor cell lines Skov-3 (ovarian), HeLa (cervical), and A549 (lung) were plated in 96-well plates at densities of 4,000 cells per well (SKOV-3 & HeLa) or 8,000 cells per well (A549), allowed to adhere for 24 hours, and treated with various concentrations of the triphenylmethane compounds for 24 hours. Cells were fixed in 4% formaldehyde and stained with antitubulin antibodies (subsequently recognized using fluorescently-labeled secondary antibody) and Hoechst dye (which stains DNA).

[0076] Visual inspection revealed that the triphenylmethane compounds caused cell cycle arrest in the prometaphase stage of mitosis. DNA was condensed and spindle formation had initiated, but arrested cells uniformly displayed monopolar spindles, indicating that there was an inhibition of spindle pole body separation. Microinjection of anti-KSP antibodies also causes mitotic arrest with arrested cells displaying monopolar spindles.

Inhibition of Cellular Proliferation in Tumor Cell Lines Treated with Triphenylmethane KSP Inhibitors.

[0077] Cells were plated in 96-well plates at densities from 1000-2500 cells/well of a 96-well plate (depending on the cell line) and allowed to adhere/grow for 24 hours. They were then treated with various concentrations of drug for 48 hours. The time at which compounds are added is considered T_0. A tetrazolium-based assay using the reagent 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (I.S> Patent No. 5,185,450) (see Promega product catalog #G3580, CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay) was used to determine the number of viable cells at T_0 and the number of cells remaining after 48 hours compound exposure. The number of cells remaining after 48 hours was compared to the number of viable cells at the time of drug addition, allowing for calculation of growth inhibition.

[0078] The growth over 48 hours of cells in control wells that had been treated with vehicle only (0.25% DMSO) is considered 100% growth and the growth of cells in

wells with compounds is compared to this. Triphenylmethane KSP inhibitors inhibited cell proliferation in human tumor cell lines of the following tumor types: lung (NCI-H460, A549), breast (MDA-MB-231, MCF-7, MCF-7/ADR-RES), colon (HT29, HCT15), ovarian (SKOV-3, OVCAR-3), leukemia (HL-60(TB), K-562), central nervous system (SF-268), renal (A498), osteosarcoma (U2-OS), and cervical (HeLa). In addition, a mouse tumor line (B16, melanoma) was also growth-inhibited in the presence of the triphenylmethane compounds.

[0079] A Gi_{50} was calculated by plotting the concentration of compound in μM vs the percentage of cell growth of cell growth in treated wells. The Gi_{50} calculated for the compounds is the estimated concentration at which growth is inhibited by 50% compared to control, i.e., the concentration at which:

$$100 \times ((Treated_{48} - T_0) / (Control_{48} - T_0)) = 50.$$

[0080] All concentrations of compounds are tested in duplicate and controls are averaged over 12 wells. A very similar 96-well plate layout and Gi_{50} calculation scheme is used by the National Cancer Institute (see Monks, et al., J. NatI. Cancer Inst. 83:757-766 (1991)). However, the method by which the National Cancer Institute quantitates cell number does not use MTS, but instead employs alternative methods.

Calculation Of IC_{50}:

[0081] Measurement of a composition's IC_{50} for KSP activity uses an ATPase assay. The following solutions are used: Solution 1 consists of 3 mM phosphoenolpyruvate potassium salt (Sigma P-7127), 2 mM ATP (Sigma A-3377), 1 mM IDTT (Sigma D-9779), 5 μM paclitaxel (Sigma T-7402), 10 ppm antifoam 289 (Sigma A-8436), 25 mM Pipes/KOH pH 6.8 (Sigma P6757), 2 mM MgC12 (VWR JT400301), and 1 mM EGTA (Sigma E3889). Solution 2 consists of 1 mM NADH (Sigma N8129), 0.2 mg/ml BSA (Sigma A7906), pyruvate kinase 7U/ml, L-lactate dehydrogenase 10 U/ml (Sigma P0294), 100 nM KSP motor domain, 50 μg/ml microtubules, 1 mM DTT (Sigma D9779), 5 μM paclitaxel (Sigma T-7402), 10 ppm antifoam 289 (Sigma A-8436), 25 mM Pipes/KOH pH 6.8 (Sigma P6757), 2 mM MgC12 (VWR JT4003-01), and 1 mM EGTA (Sigma E3889). Serial dilutions (8-12 two-fold dilutions) of the composition are made in a 96-well microtiter plate (Corning

Costar 3695) using Solution 1. Following serial dilution each well has 50 µl of Solution 1. The reaction is started by adding 50 µl of solution 2 to each well. This may be done with a multichannel pipettor either manually or with automated liquid handling devices. The microtiter plate is then transferred to a microplate absorbance reader and multiple absorbance readings at 340 nm are taken for each well in a kinetic mode. The observed rate of change, which is proportional to the ATPase rate, is then plotted as a function of the compound concentration. For a standard IC_{50} determination the data acquired is fit by the following four parameter equation using a nonlinear fitting program (e.g., Grafit 4):

$$y = \frac{\text{Range}}{1 + \left(\frac{x}{IC_{50}}\right)^{s}} + \text{Background}$$

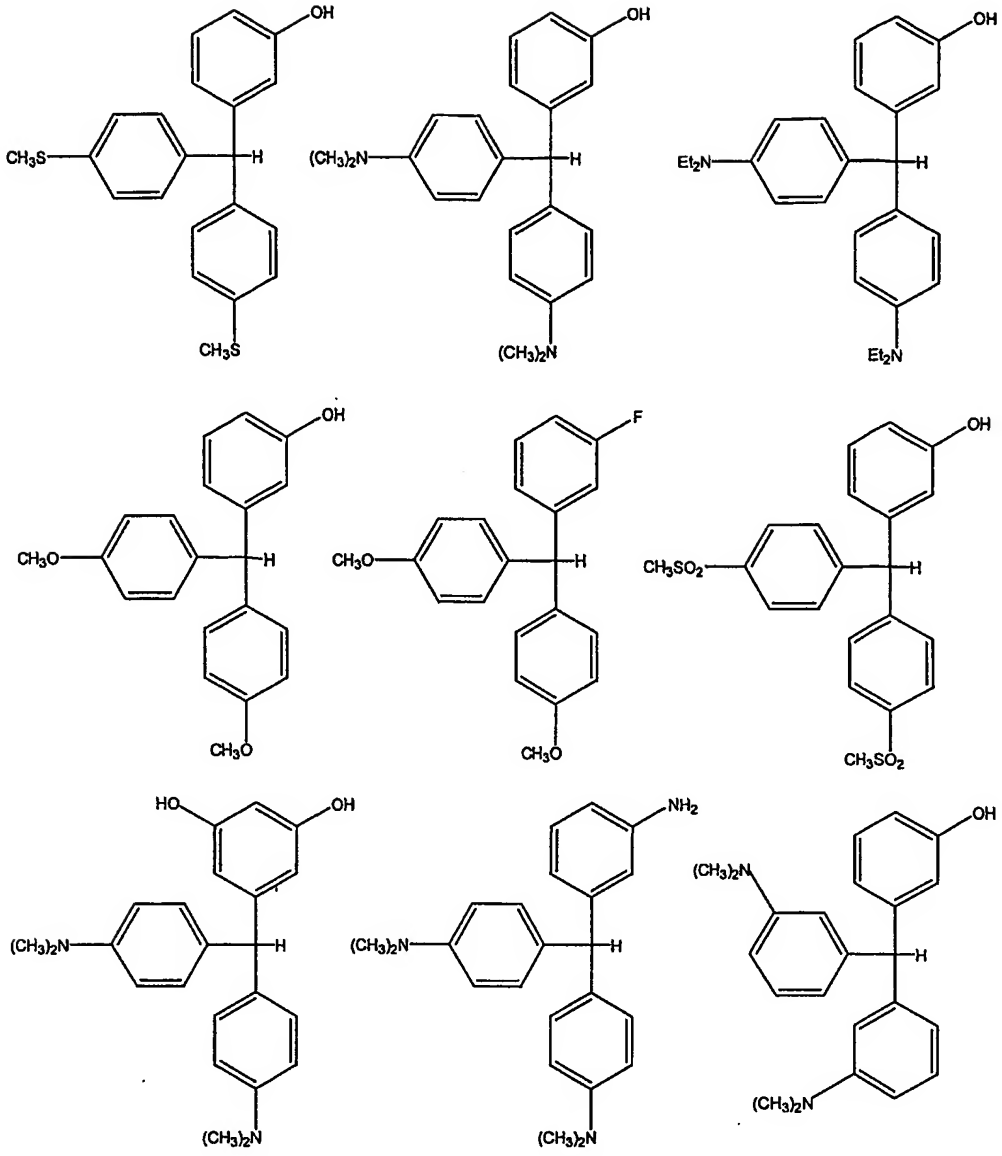
where y is the observed rate and x the compound concentration.

[0082] The K_i for a compound is determined from the IC_{50} based on three assumptions. First, only one compound molecule binds to the enzyme and there is no cooperativity. Second, the concentrations of active enzyme and the compound tested are known (i.e., there are no significant amounts of impurities or inactive forms in the preparations). Third, the enzymatic rate of the enzyme-inhibitor complex is zero. The rate (i.e., compound concentration) data are fitted to the equation:

$$V = V_{max} E_0 \left[I - \frac{(E_0 + I_0 + Kd) - \sqrt{(E_0 + I_0 + Kd)^2 - 4 E_0 I_0}}{2E_0} \right]$$

where V is the observed rate, V_{max} is the rate of the free enzyme, I_0 is the inhibitor concentration, E_0 is the enzyme concentration, and K_d is the dissociation constant of the enzyme-inhibitor complex.

[0083] Several representative compounds of the invention were tested as described above and found to exhibit Ki's below 100 µM. Their structures are as shown:


OH
CH3S
H
CH3S
OH
(CH3)2N
H
(CH3)2N
OH
Et2N
H
Et2N
OH
CH3O
H
CH3O
F
CH3O
H
CH3O
OH
CH3SO2
H
CH3SO2
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OH
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OH
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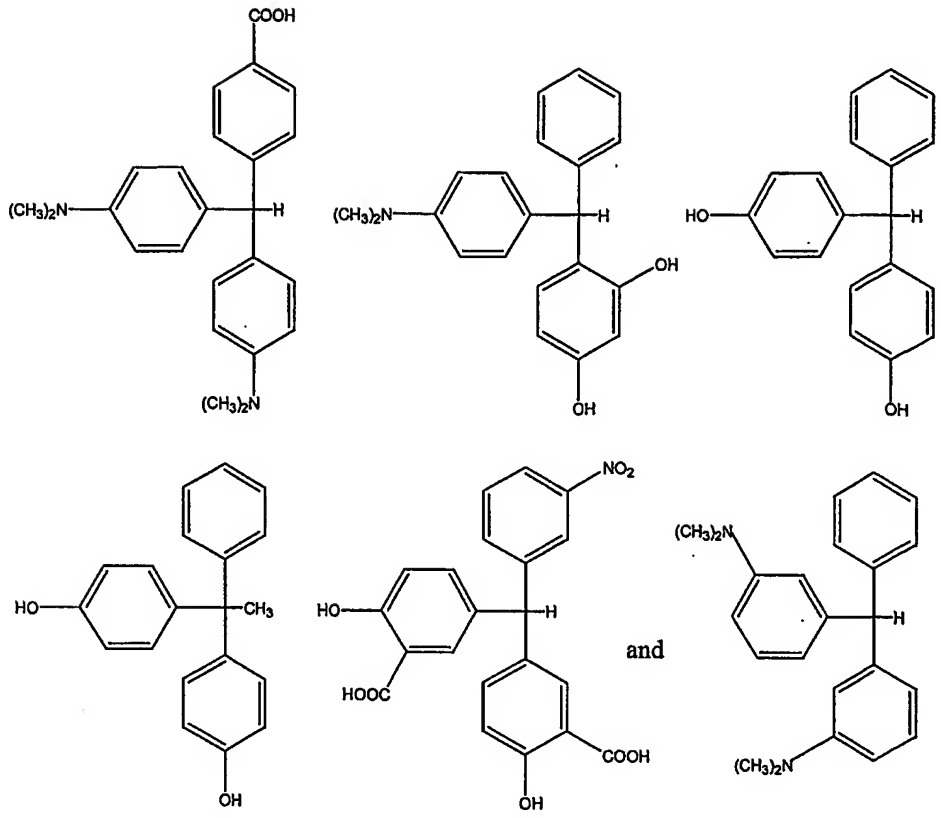
H
(CH3)2N



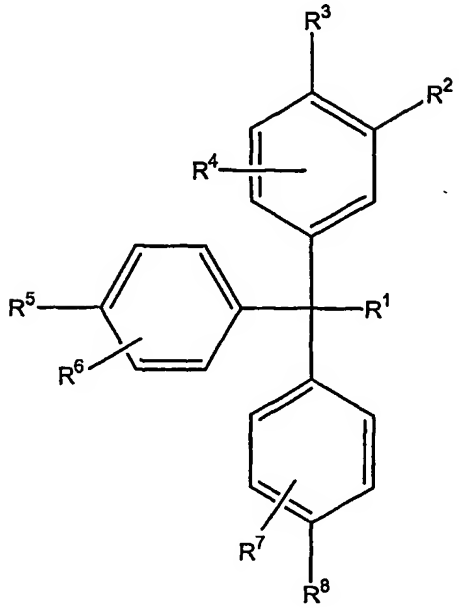
[0084] The triphenylmethane compounds inhibit growth in a variety of cell lines, including cell lines (MCF-7/ADR-RES, HCT1 5) that express P-glycoprotein (also known as Multi-drug Resistance, or MDR^+), which conveys resistance to other chemotherapeutic drugs, such as pacilitaxel. Therefore, the triphenylmethanes are anti-mitotics that inhibit cell proliferation, and are not subject to resistance by overexpression of MDR^+ by drug-resistant tumor lines.

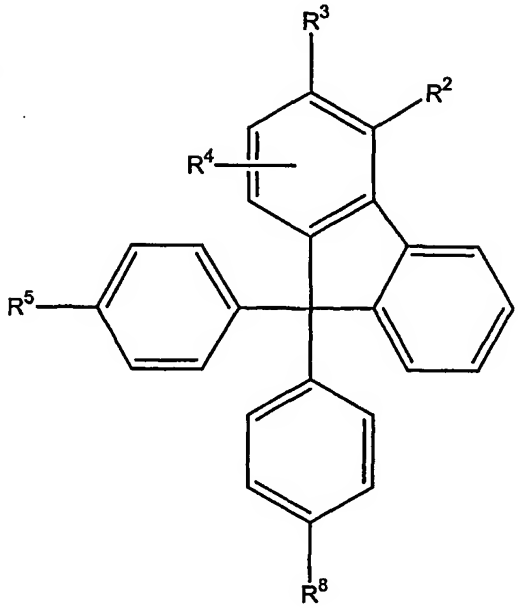
[0085] Compounds of this class were found to inhibit cell proliferation, although GI_{50} values varied. GI_{50} values for the triphenylmethane compounds tested ranged from 200 nM to greater than the highest concentration tested. By this we mean that although most of the compounds that inhibited KSP activity biochemically did inhibit cell proliferation, for some, at the highest concentration tested (generally about 20 μM), cell growth was inhibited less than 50%. Many of the compounds have GI_{50} values less than 10 μM, and several have GI_{50} values less than 1 μM. Anti-proliferative compounds that have been successfully applied in the clinic to treatment of cancer (cancer chemotherapeutics) have GI_{50}'s that vary greatly. For example, in A549 cells, paclitaxel GI_{50} is 4 nM, doxorubicin is 63 nM, 5-fluorouracil is 1 μM, and hydroxyurea is 500 μM

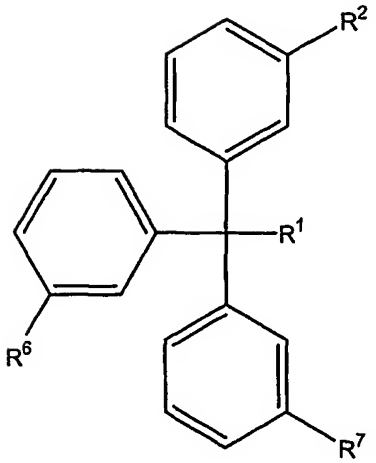
(data provided by National Cancer Institute, Developmental Therapeutic Program, http://dtp.nci.nih.gov/). Therefore, compounds that inhibit cellular proliferation at virtually any concentration may be useful. However, preferably, compounds will have GI_{50} values of less than 1 mM. More preferably, compounds will have GI_{50} values of less than 20 μM. Even more preferably, compounds will have GI_{50} values of less than 10 μM. Further reduction in GI_{50} values may also be desirable, including compounds with GI_{50} values of less than 1 μM.

We claim:

1. A method of treating cellular proliferative diseases comprising administering a compound chosen from:







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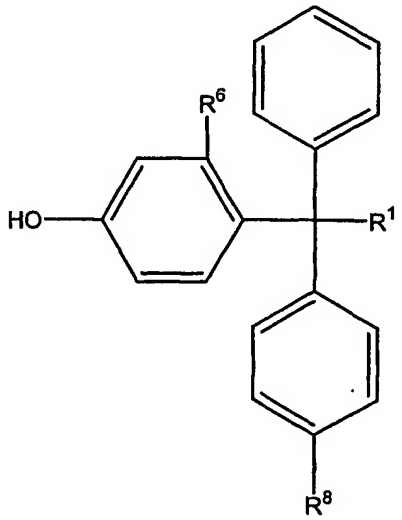


wherein

R^1 is hydrogen or lower alkyl;

R^2 is chosen from H, -OH, -F, $-NH_2$, and $-NO_2$;

R^3 is chosen from H, -COOH, -O-(alkyl), and -OH;

R^4 is chosen from H, -OH, and -COO(alkyl);

R^5 is chosen from -S-(alkyl), $-NH_2$, $-N(alkyl)_2$, -OH, -O-(alkyl) and SO_2CH_3;

R^6 is chosen from H, $-N(alkyl)_2$, -OH and -COOH;

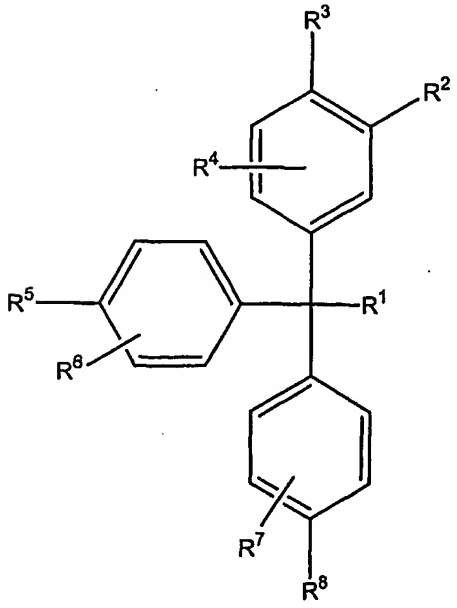
R^7 is chosen from H, $-N(alkyl)_2$, -OH and -COOH;

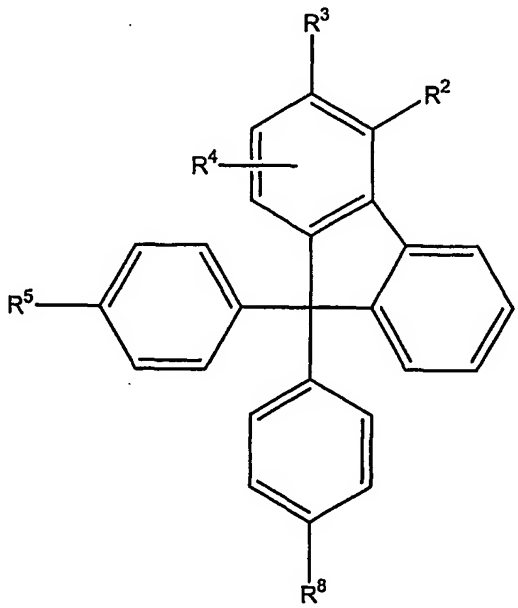
R^8 is chosen from -S-(alkyl), $-NH_2$, $-N(alkyl)_2$, -OH, -O-(alkyl) and SO_2CH_3; and

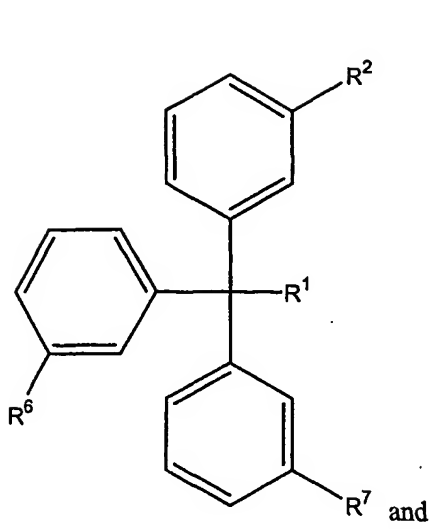
or a pharmaceutically acceptable salt thereof,

with the proviso that at least one of R^2, R^3 and R^4 must be other than hydrogen.

2. A method of treating a disorder associated with KSP kinesin activity comprising administering a compound chosen from:







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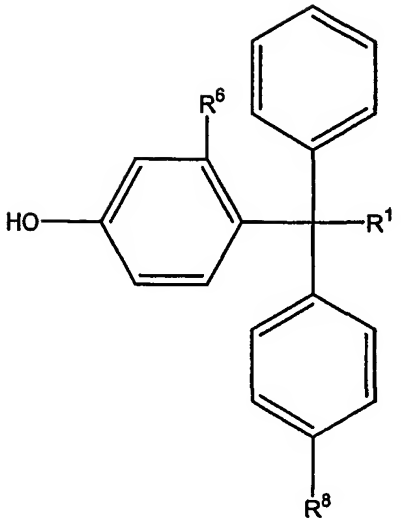


wherein

R^1 is hydrogen or lower alkyl;

R^2 is chosen from H, -OH, -F, $-NH_2$, and $-NO_2$;

R^3 is chosen from H, -COOH, -O-(alkyl), and -OH;

R^4 is chosen from H, -OH, and -COO(alkyl);

R^5 is chosen from -S-(alkyl), $-NH_2$, $-N(alkyl)_2$, -OH, -O-(alkyl) and SO_2CH_3;

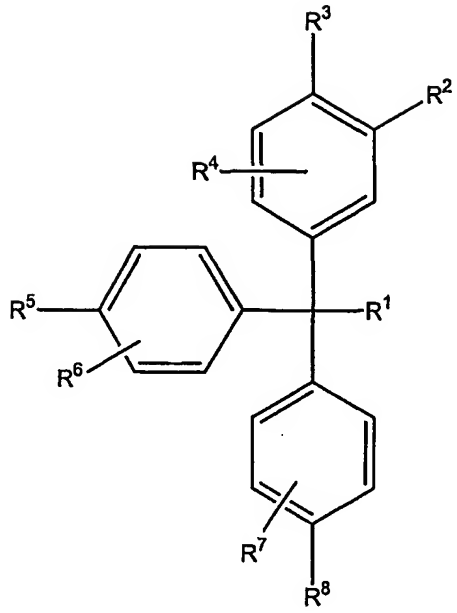
R^6 is chosen from H, $-N(alkyl)_2$, -OH and -COOH;

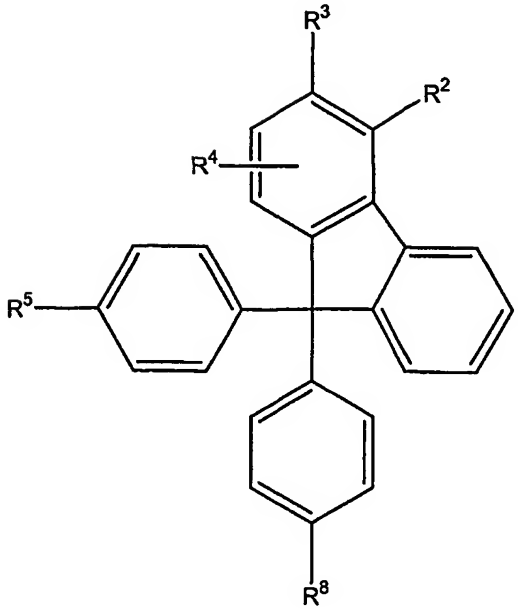
R^7 is chosen from H, $-N(alkyl)_2$, -OH and -COOH;

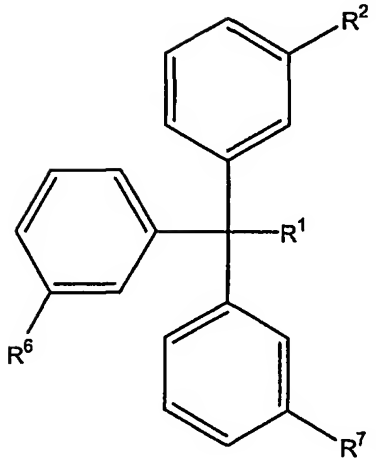
R^8 is chosen from -S-(alkyl), $-NH_2$, $-N(alkyl)_2$, -OH, -O-(alkyl) and SO_2CH_3;

or a pharmaceutically acceptable salt thereof, with the proviso that at least one of R^2, R^3 and R^4 must be other than hydrogen.

3. A method of inhibiting KSP kinesin comprising contacting KSP kinesin with a compound chosen from:







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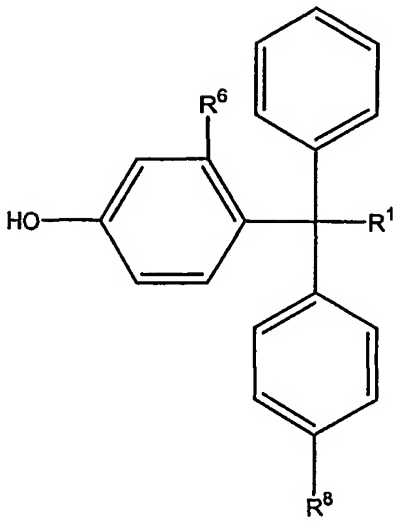


wherein

R^1 is hydrogen or lower alkyl;

R^2 is chosen from H, -OH, -F, $-NH_2$, and $-NO_2$;

R^3 is chosen from H, -COOH, -O-(alkyl), and -OH;

R^4 is chosen from H, -OH, and -COO(alkyl);

R^5 is chosen from -S-(alkyl), $-NH_2$, $-N(alkyl)_2$, -OH, -O-(alkyl) and SO_2CH_3;

R^6 is chosen from H, $-N(alkyl)_2$, -OH and -COOH;

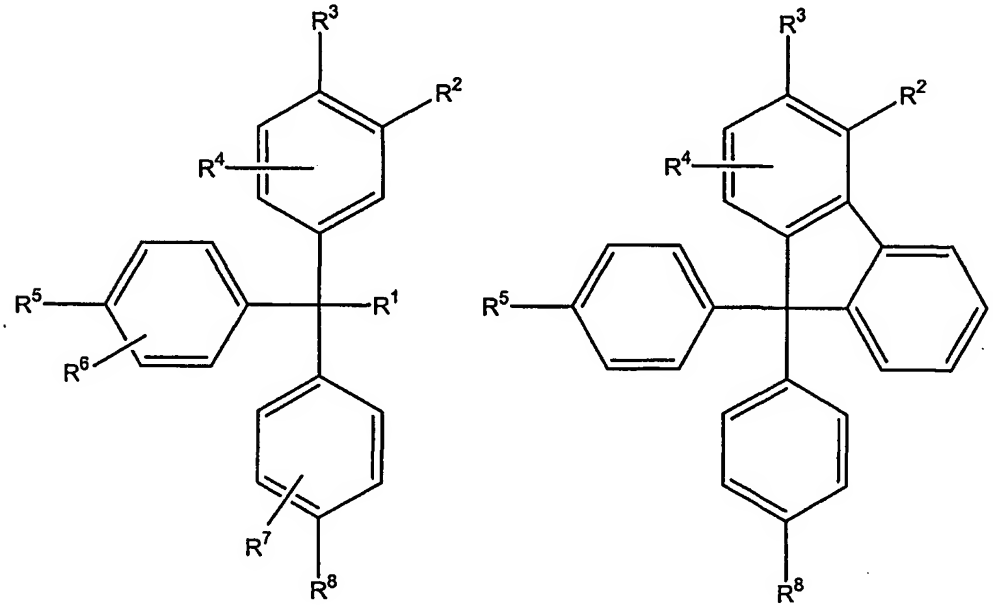
R^7 is chosen from H, $-N(alkyl)_2$, -OH and -COOH;

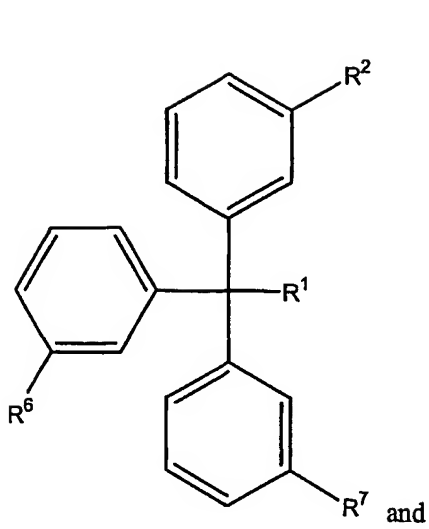
R^8 is chosen from -S-(alkyl), $-NH_2$, $-N(alkyl)_2$, -OH, -O-(alkyl) and SO_2CH_3;

or a pharmaceutically acceptable salt thereof, with the proviso that at least one of R^2, R^3 and R^4 must be other than hydrogen.

4. A method of screening for KSP kinesin modulators comprising:

combining a kinesin, a candidate bioactive agent and a compound chosen from:





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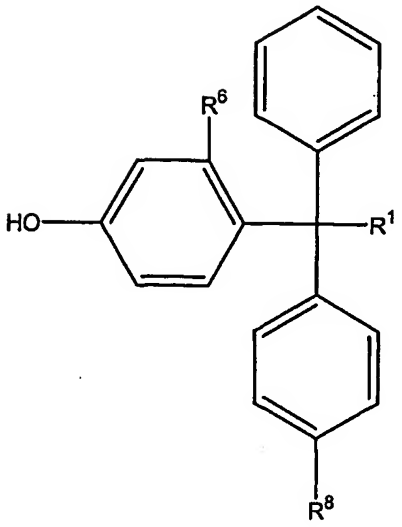


wherein

R^1 is hydrogen or lower alkyl;

R^2 is chosen from H, -OH, -F, $-NH_2$, and $-NO_2$;

R^3 is chosen from H, -COOH, -O-(alkyl), and -OH;

R^4 is chosen from H, -OH, and -COO(alkyl);

R^5 is chosen from -S-(alkyl), $-NH_2$, $-N(alkyl)_2$, -OH, -O-(alkyl) and SO_2CH_3;

R^6 is chosen from H, $-N(alkyl)_2$, -OH and -COOH;

R^7 is chosen from H, $-N(alkyl)_2$, -OH and -COOH;

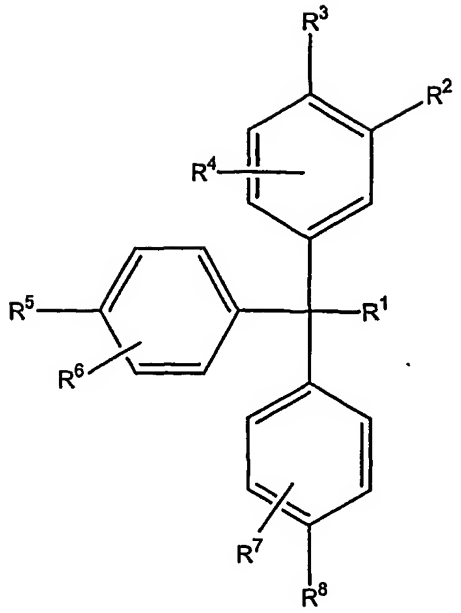
R^8 is chosen from -S-(alkyl), $-NH_2$, $-N(alkyl)_2$, -OH, -O-(alkyl) and SO_2CH_3;

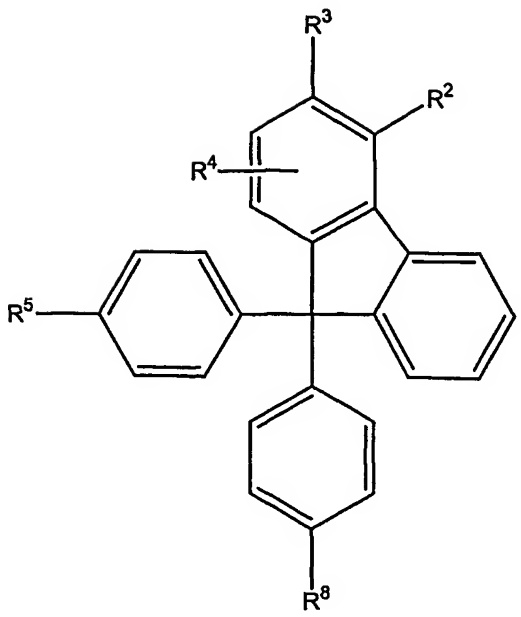
or a pharmaceutically acceptable salt thereof, with the proviso that at least one of R^2, R^3 and R^4 must be other than hydrogen, and

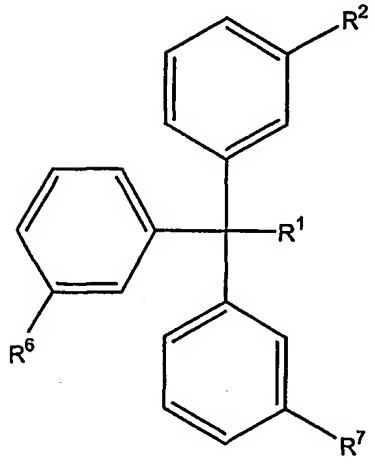
determining the effect of said candidate bioactive agent on the activity of said kinesin.

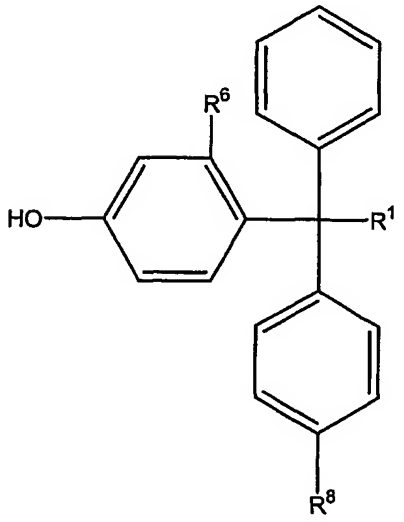
5. A method of screening for compounds that bind to KSP kinesin comprising:

combining a kinesin, a candidate bioactive agent and a labeled compound chosen from:









and

wherein

R^1 is hydrogen or lower alkyl;

R^2 is chosen from H, -OH, -F, $-NH_2$, and $-NO_2$;

R^3 is chosen from H, -COOH, -O-(alkyl), and -OH;

R^4 is chosen from H, -OH, and -COO(alkyl);

R^5 is chosen from -S-(alkyl), $-NH_2$, $-N(alkyl)_2$, -OH, -O-(alkyl) and SO_2CH_3;

R^6 is chosen from H, $-N(alkyl)_2$, -OH and -COOH;

R^7 is chosen from H, $-N(alkyl)_2$, -OH and -COOH;

R^8 is chosen from -S-(alkyl), $-NH_2$, $-N(alkyl)_2$, -OH, -O-(alkyl) and SO_2CH_3; or a pharmaceutically acceptable salt thereof, with the proviso that at least one of R^2, R^3 and R^4 must be other than hydrogen; and

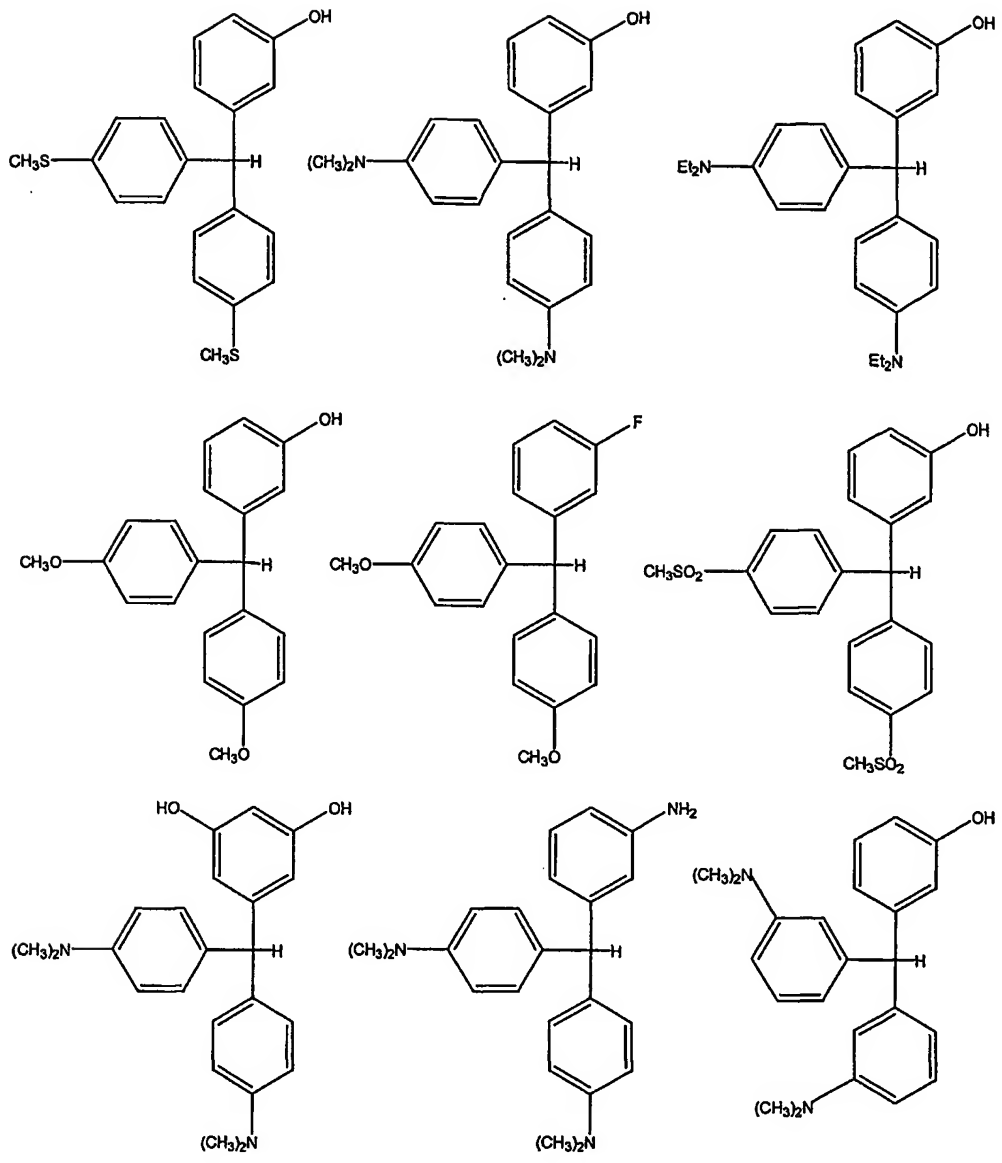
determining the binding of said candidate bioactive agent to said kinesin.

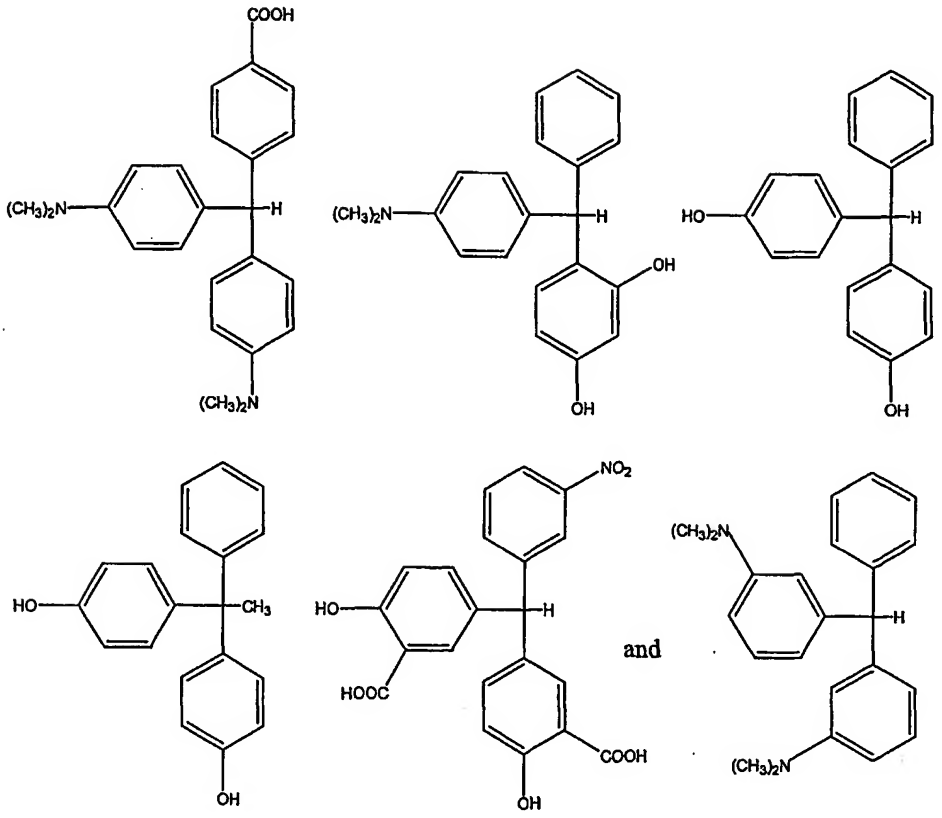
6. A method according to any of claims 1 to 5 wherein R^2 is chosen from -OH, -F, $-NH_2$, and $-NO_2$.

7. A method according to any of claims 1 to 5 wherein R^2 is H and R^3 is -OH, -COOH or $-OCH_3$.

8. A method according to any of claims 1 to 5 wherein R^5 and R^8 are chosen from $-N(alkyl)_2$ and -OH.

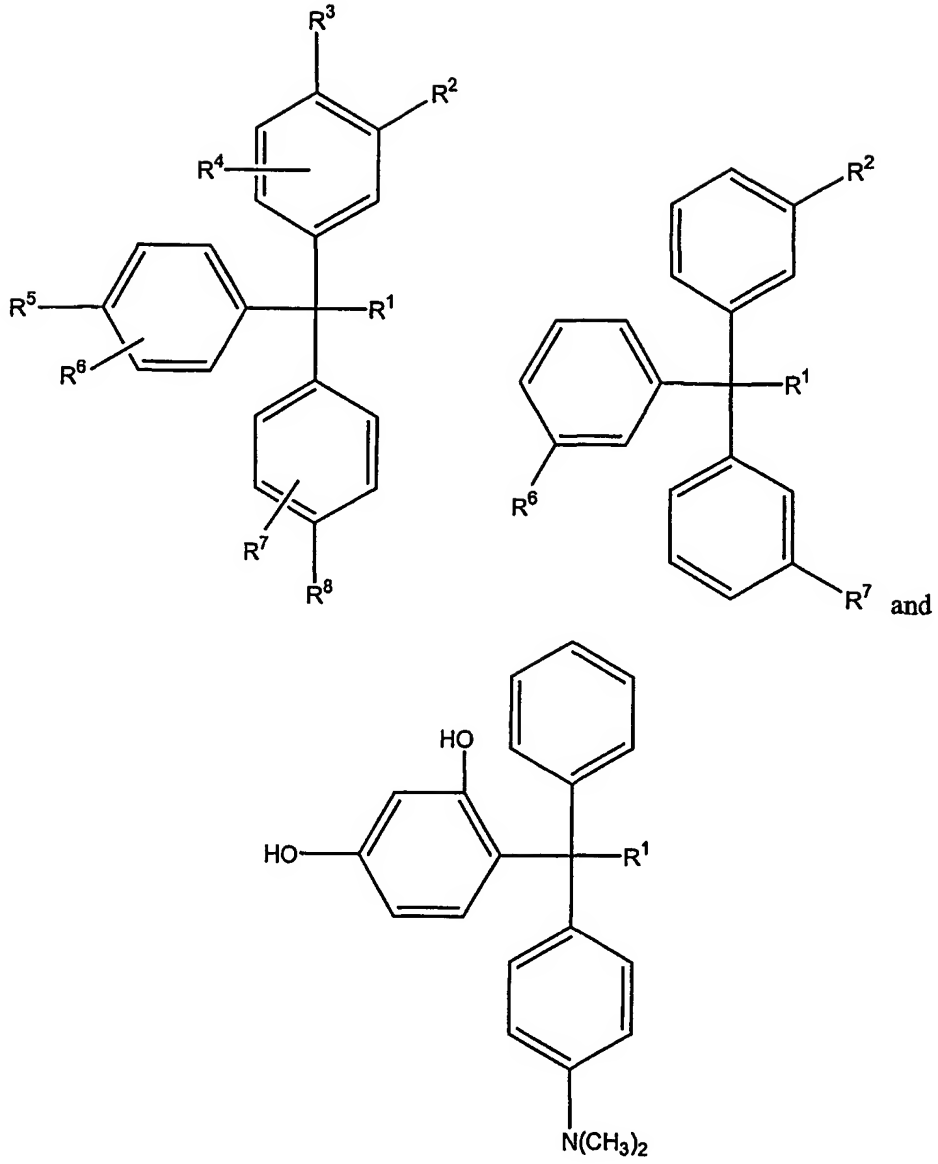
9. A method according to any of claims 1 to 5 wherein said compound is chosen from





10. A method according to claim 1 or 2 wherein said disease or disorder is chosen from the group consisting of cancer, hyperplasia, restenosis, cardiac hypertrophy, immune disorders and inflammation.

11. A triphenylmethane chosen from



wherein

R^1 is hydrogen or lower alkyl;

R^2 is chosen from H, -OH, -F, $-NH_2$, and $-NO_2$;

R^3 is chosen from H, -COOH, -O-(alkyl), and -OH;

R^4 is chosen from H, -OH, and -COO(alkyl);

R^5 is chosen from -S-(alkyl), $-NH_2$, $-N(alkyl)_2$, -OH, -O-(alkyl) and SO_2CH_3;

R^6 is chosen from H, -N(alkyl)$_2$, -OH and -COOH;

R^7 is chosen from H, -N(alkyl)$_2$, -OH and -COOH; and

R^8 is chosen from -S-(alkyl), -NH_2, -N(alkyl)$_2$, -OH, -O-(alkyl) and SO_2CH_3;

with the provisos that at least one of R^2, R^3 and R^4 must be other than hydrogen and, when R^2 is -OH and R^3 and R^4 are hydrogen, R^5 and R^8 cannot be -N$(CH_3)_2$, or a pharmaceutically acceptable salt thereof

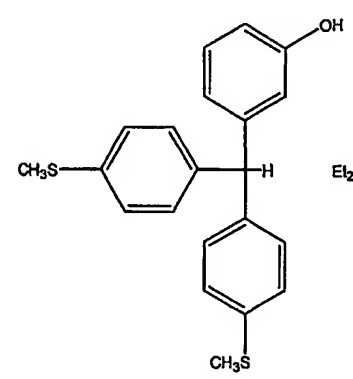
12. A triphenylmethane according to claim 11 wherein R^2 is chosen from -OH, -F, and -NH_2.

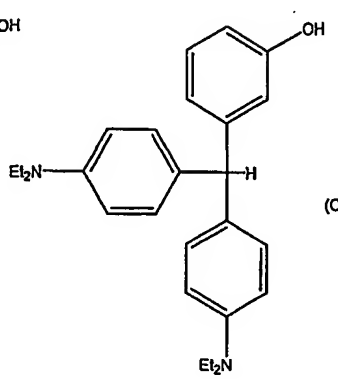
13. A triphenylmethane according to claim 12 wherein R^6 and R^7 are hydrogen.

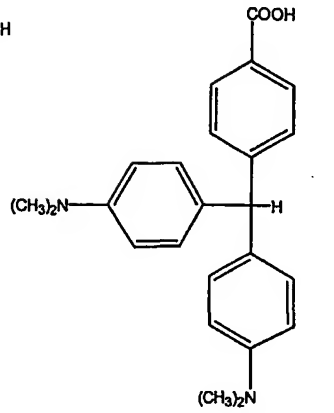
14. A triphenylmethane according to claim 12 wherein R^6 and R^7 are -N(alkyl)$_2$.

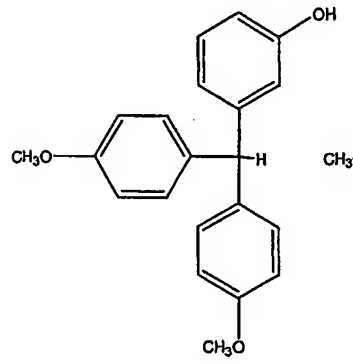
15. A triphenylmethane according to claim 11 wherein R^5 and R^8 chosen from -S-CH_3, -N(lower-alkyl)$_2$ and SO_2CH_3.

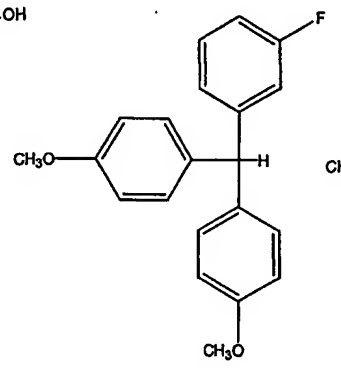
16. A triphenylmethane according to claim 11 chosen from

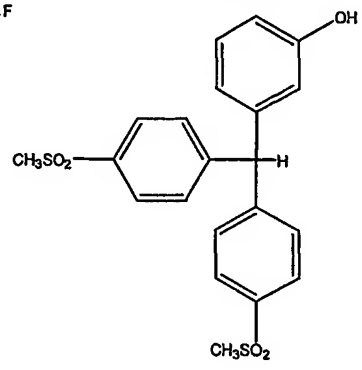


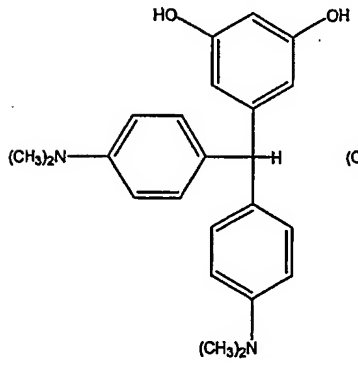


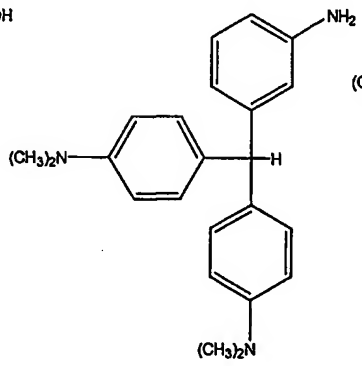


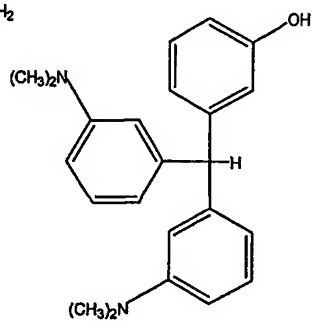


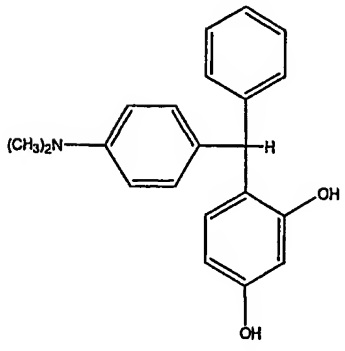


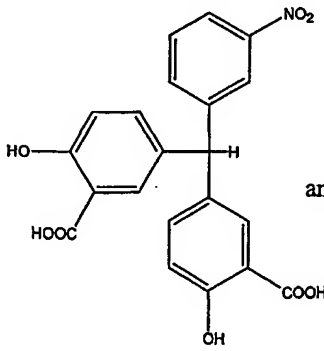












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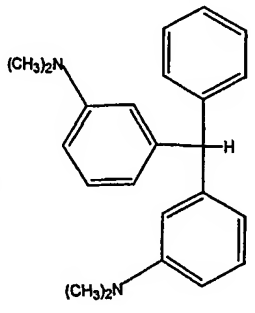

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 02/01614

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/135 A61K31/10 A61P9/00 A61P29/00 A61P35/00
A61P37/00 A61P43/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, CHEM ABS Data, MEDLINE, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 69439 A (OLESEN SOEREN PETER ;NEUROSEARCH AS (DK); CHRISTOPHERSEN PALLE (DK) 23 November 2000 (2000-11-23) abstract page 4, line 20 -page 14, line 28 page 19, line 21 -page 20, line 17 page 21, line 5 - line 37 claims 1-25 ---	11-16
X	WO 00 00468 A (UNIV CALIFORNIA) 6 January 2000 (2000-01-06) abstract page 5, line 9 -page 8, line 1 claims 1-39 --- -/--	11-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 May 2002	29/05/2002

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 06137 A (ABBOTT LAB) 10 February 2000 (2000-02-10) abstract page 3, line 13 -page 12, line 25 claims 1-19	11-16
X	EP 1 000 925 A (SUMITOMO CHEMICAL CO) 17 May 2000 (2000-05-17) page 2, line 26 -page 4, line 37 claims 1-7	11-16
X	US 6 166 013 A (COGHLAN MICHAEL J ET AL) 26 December 2000 (2000-12-26) abstract claims 1-9	11-16
P,A	WO 01 31335 A (BERAUD CHRISTOPHE ;FINER JEFFREY T (US); MAK JOHN (US); SAKOWICZ R) 3 May 2001 (2001-05-03) abstract claims 1-59	4-10
A	MUTHYALA, R; KATRITZKY, A R; LAN, X: "A Synthetic Study on the Preparation of Triarylmethanes" DYES & PIGMENTS, vol. 25, 1994, pages 303-324, XP001078895 abstract	11-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 02/01614

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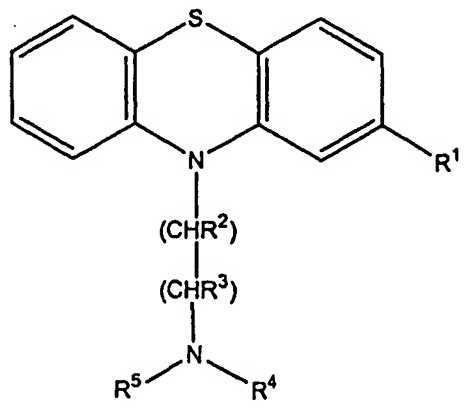
(54) Title: PHENOTHIAZINE KINESIN INHIBITORS



(I)

(57) Abstract: Phenothiazine derivatives of formula (I) are disclosed. The compounds are inhibitors of the mitotic kinesin KSP and are useful in the treatment of cellular proliferative diseases, such as cancer, hyperplasias, restenosis, cardiac hypertrophy, immune disorders and inflammation.

WO 02/057244 A1

PHENOTHIAZINE KINESIN INHIBITORS

RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. §119(e) to USSN 60/263,092, filed January 19, 2001, which is incorporated by reference in their entirety for all purposes.

FIELD OF THE INVENTION

[0002] This invention relates to phenothiazine derivatives which are inhibitors of the mitotic kinesin KSP and are useful in the treatment of cellular proliferative diseases, for example cancer, hyperplasias, restenosis, cardiac hypertrophy, immune disorders and inflammation.

BACKGROUND OF THE INVENTION

[0003] Among the therapeutic agents used to treat cancer are the taxanes and vinca alkaloids, which act on microtubules. Microtubules are the primary structural element of the mitotic spindle. The mitotic spindle is responsible for distribution of replicate copies of the genome to each of the two daughter cells that result from cell division. It is presumed that disruption of the mitotic spindle by these drugs results in inhibition of cancer cell division, and induction of cancer cell death. However, microtubules form other types of cellular structures, including tracks for intracellular transport in nerve processes. Because these agents do not specifically target mitotic spindles, they have side effects that limit their usefulness.

[0004] Improvements in the specificity of agents used to treat cancer is of considerable interest because of the therapeutic benefits which would be realized if the side effects associated with the administration of these agents could be reduced. Traditionally, dramatic improvements in the treatment of cancer are associated with identification of therapeutic agents acting through novel mechanisms. Examples of this include not only the taxanes, but also the camptothecin class of topoisomerase I inhibitors. From both of these perspectives, mitotic kinesins are attractive targets for new anti-cancer agents.

[0005] Mitotic kinesins are enzymes essential for assembly and function of the mitotic spindle, but are not generally part of other microtubule structures, such as in nerve processes. Mitotic kinesins play essential roles during all phases of mitosis. These

enzymes are "molecular motors" that transform energy released by hydrolysis of ATP into mechanical force which drives the directional movement of cellular cargoes along microtubules. The catalytic domain sufficient for this task is a compact structure of approximately 340 amino acids. During mitosis, kinesins organize microtubules into the bipolar structure that is the mitotic spindle. Kinesins mediate movement of chromosomes along spindle microtubules, as well as structural changes in the mitotic spindle associated with specific phases of mitosis. Experimental perturbation of mitotic kinesin function causes malformation or dysfunction of the mitotic spindle, frequently resulting in cell cycle arrest and cell death.

[0006] Among the mitotic kinesins which have been identified is KSP. KSP belongs to an evolutionarily conserved kinesin subfamily of plus end-directed microtubule motors that assemble into bipolar homotetramers consisting of antiparallel homodimers. During mitosis KSP associates with microtubules of the mitotic spindle. Microinjection of antibodies directed against KSP into human cells prevents spindle pole separation during prometaphase, giving rise to monopolar spindles and causing mitotic arrest and induction of programmed cell death. KSP and related kinesins in other, non-human, organisms, bundle antiparallel microtubules and slide them relative to one another, thus forcing the two spindle poles apart. KSP may also mediate in anaphase B spindle elongation and focussing of microtubules at the spindle pole.

[0007] Human KSP (also termed HsEg5) has been described (Blangy, et al., Cell, 83:1159-69 (1995); Whitehead, et al., Arthritis Rheum., 39:1635-42 (1996); Galgio et al., J. Cell Biol., 135:339-414 (1996); Blangy, et al., J Biol. Chem., 272:19418-24 (1997); Blangy, et al., Cell Motil Cytoskeleton, 40:174-82 (1998); Whitehead and Rattner, J. Cell Sci., 111:2551-61 (1998); Kaiser, et al., JBC 274:18925-31 (1999); GenBank accession numbers: X85137, NM004523 and U37426) , and a fragment of the KSP gene (TRIP5) has been described (Lee, et al., Mol Endocrinol., 9:243-54 (1995); GenBank accession number L40372). Xenopus KSP homologs (Eg5), as well as Drosophila KLP61 F/KRP1 30 have been reported.

[0008] Mitotic kinesins are attractive targets for the discovery and development of novel mitotic chemotherapeutics. Accordingly, it is an object of the present invention to provide methods and compositions useful in the inhibition of KSP, a mitotic kinesin.

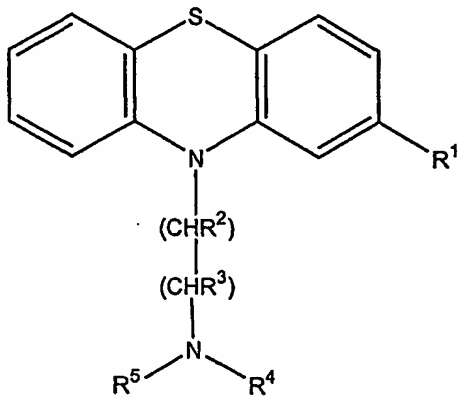
[0009] Phenothiazines have been known as psychopharmacologic agents for many years. Chlorpromazine, fluphenazine, perphenazine, trifluoperazine, promazine

and thioridazine are typical examples. Inhibition of KSP by phenothiazines has not been described.

SUMMARY OF THE INVENTION

[0010] In accordance with the objects outlined above, the present invention provides compositions and methods that can be used to treat diseases of proliferating cells. The compositions are KSP inhibitors, particularly human KSP inhibitors.

[0011] In one aspect, the invention relates to methods for treating cellular proliferative diseases, for treating disorders associated with KSP kinesin activity, and for inhibiting KSP kinesin. The methods employ compounds of the formula:



wherein

R^1 is hydrogen, halogen or CF_3;

R^2 is chosen from hydrogen and lower alkyl;

R^3 is hydrogen;

R^4 and R^5 are independently chosen from hydrogen, alkyl, substituted alkyl, alkylaryl, substituted alkylaryl, alkylheteroaryl and substituted alkylheteroaryl; or

any of R^2, R^3 and R^4 taken together with the intervening atoms form one or more five- to seven-membered rings, or a pharmaceutically acceptable salt thereof.

[0012] The ring may be substituted with one or more alkyl, aryl, alkoxy, halo, alkylaryl or substituted alkylaryl substituents. It is necessary for activity that the phenothiazine contain at least one five- to seven-membered ring in addition to the three rings of the phenothiazine.

[0013] Diseases and disorders that respond to therapy with compounds of the invention include cancer, hyperplasia, restenosis, cardiac hypertrophy, immune disorders and inflammation.

[0014] In another aspect, the invention relates to compounds useful in inhibiting KSP kinesin. The compounds have the structures shown above.

[0015] In an additional aspect, the present invention provides methods of screening for compounds that will bind to a KSP kinesin, for example compounds that will displace or compete with the binding of the compositions of the invention. The methods comprise combining a labeled compound of the invention, a KSP kinesin, and at least one candidate agent and determining the binding of the candidate bioactive agent to the KSP kinesin.

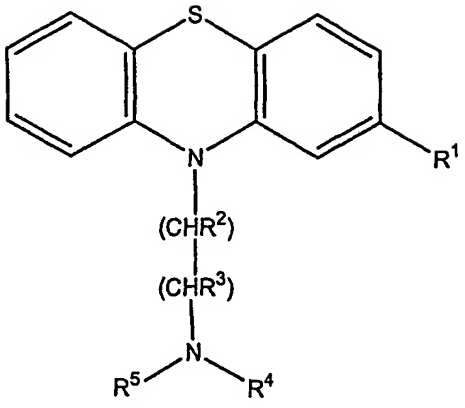
[0016] In a further aspect, the invention provides methods of screening for modulators of KSP kinesin activity. The methods comprise combining a composition of the invention, a KSP kinesin, and at least one candidate agent and determining the effect of the candidate bioactive agent on the KSP kinesin activity.

DETAILED DESCRIPTION OF THE INVENTION

[0017] The present invention is directed to a class of novel phenothiazines that are modulators of mitotic kinesins. By inhibiting or modulating mitotic kinesins, but not other kinesins (e.g., transport kinesins), specific inhibition of cellular proliferation is accomplished. Thus, the present invention capitalizes on the finding that perturbation of mitotic kinesin function causes malformation or dysfunction of mitotic spindles, frequently resulting in cell cycle arrest and cell death. The methods of inhibiting a human KSP kinesin comprise contacting an inhibitor of the invention with a KSP kinesin, particularly human KSP kinesins, including fragments and variants of KSP. The inhibition can be of the ATP hydrolysis activity of the KSP kinesin and/or the mitotic spindle formation activity, such that the mitotic spindles are disrupted. Meiotic spindles may also be disrupted.

[0018] An object of the present invention is to develop inhibitors and modulators of mitotic kinesins, in particular KSP, for the treatment of disorders associated with cell proliferation. Traditionally, dramatic improvements in the treatment of cancer, one type of cell proliferative disorder, have been associated with identification of therapeutic agents acting through novel mechanisms. Examples of this include not only the taxane class of agents that appear to act on microtubule formation, but also the camptothecin class of topoisomerase I inhibitors. The compositions and methods described herein can differ in their selectivity and are preferably used to treat diseases of proliferating cells, including, but not limited to cancer, hyperplasias, restenosis, cardiac hypertrophy, immune disorders and inflammation.

[0019] Accordingly, the present invention relates to methods employing phenothiazines of the formula:



wherein

R^1 is hydrogen, halogen or CF_3;

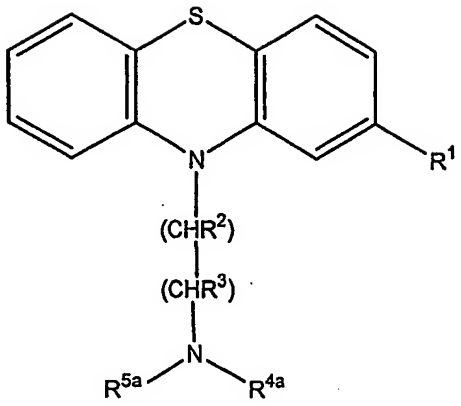
R^2 is chosen from hydrogen and lower alkyl;

R^3 is hydrogen;

R^4 and R^5 are independently chosen from hydrogen, alkyl, substituted alkyl, alkylaryl, substituted alkylaryl, alkylheteroaryl and substituted alkylheteroaryl; or

any of R^2, R^3 and R^4 taken together with the intervening atoms form one or more five- to seven-membered rings that may be optionally substituted with one or more alkyl, aryl, alkoxy, halo, alkylaryl or substituted alkylaryl substituents, or a pharmaceutically acceptable salt thereof. It is necessary for activity that the phenothiazine contain at least one five- to seven-membered ring in addition to the three rings of the phenothiazine.

[0020] All of the compounds falling within the foregoing parent genus and its subgenera are useful as kinesin inhibitors, but not all the compounds are novel. In particular, certain known species fall within the genus in which R^4 and R^5 have the full breadth of operative substituents, although no utility in inhibiting kinesin has been suggested for these species. Any narrowing of the claims or specific exceptions that might be added to these claims reflect applicants' intent to avoid claiming subject matter that, while functionally part of the inventive concept, is not patentable to them for reasons having nothing to do with the scope of their invention. In particular, the novel compounds that are the subject of the claims are described by the formula:



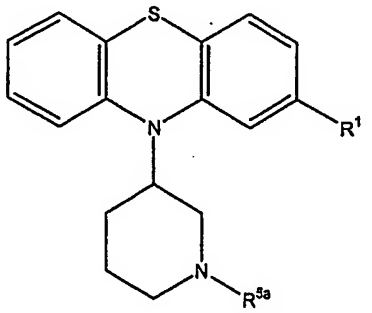
wherein R^1, R^2 and R^3 are as defined above;

R^{4a} is chosen from hydrogen and lower alkyl; and

R^{5a} is chosen from alkylaryl, substituted alkylaryl, alkylheteroaryl and substituted alkylheteroaryl; or

any of R^2, R^3 and R^{4a} taken together with the intervening atoms form one or more five- to seven-membered rings, which may be optionally substituted with one or more alkyl, aryl, alkoxy, halo, alkylaryl or substituted alkylaryl substituents, or a pharmaceutically acceptable salt thereof.

[0021] Preferred compounds of the methods and compositions are those in which R^3 is hydrogen and R^2 and R^{4a} form a five- to seven-membered ring. Such compounds include phenothiazines of formula



[0022] In most preferred compounds R^{5a} is benzyl or substituted benzyl.

[0023] Other preferred compounds of the methods and compositions are those in which R^2 and R^3 are hydrogen and R^{5a} is alkylaryl or substituted alkylaryl, particularly those in which R^{5a} is benzyl or substituted benzyl.

Definitions

[0024] Alkyl is intended to include linear, branched, or cyclic hydrocarbon structures and combinations thereof. Lower alkyl refers to alkyl groups of from 1 to 5 carbon atoms. Examples of lower alkyl groups include methyl, ethyl, propyl, isopropyl, butyl, s-and t-butyl and the like. Preferred alkyl groups are those of C_{20} or below. More preferred alkyl groups are those of C_{13} or below. Cycloalkyl is a subset of alkyl and includes cyclic hydrocarbon groups of from 3 to 13 carbon atoms. Examples of cycloalkyl groups include c-propyl, c-butyl, c-pentyl, norbornyl, adamantyl and the like. In this application, alkyl refers to alkanyl, alkenyl and alkynyl residues; it is intended to include cyclohexylmethyl, vinyl, allyl, isoprenyl and the like. Alkylene refers to the same residues as alkyl, but having two points of attachment. Examples of alkylene include ethylene ($-CH_2CH_2-$), propylene ($-CH_2CH_2CH_2-$), dimethylpropylene ($-CH_2C(CH_3)_2CH_2-$) and cyclohexylpropylene ($-CH_2CH_2CH(C_6H_{13})-$). When an alkyl residue having a specific number of carbons is named, all geometric isomers having that number of carbons are intended to be encompassed; thus, for example, "butyl" is meant to include n-butyl, sec-butyl, isobutyl and t-butyl; "propyl" includes n-propyl and isopropyl.

[0025] Alkoxy or alkoxyl refers to groups of from 1 to 8 carbon atoms of a straight, branched, cyclic configuration and combinations thereof attached to the parent structure through an oxygen. Examples include methoxy, ethoxy, propoxy, isopropoxy, cyclopropyloxy, cyclohexyloxy and the like. Lower-alkoxy refers to groups containing one to four carbons.

[0026] Acyl refers to groups of from 1 to 8 carbon atoms of a straight, branched, cyclic configuration, saturated, unsaturated and aromatic and combinations thereof, attached to the parent structure through a carbonyl functionality. One or more carbons in the acyl residue may be replaced by nitrogen, oxygen or sulfur as long as the point of attachment to the parent remains at the carbonyl. Examples include acetyl, benzoyl, propionyl, isobutyryl, t-butoxycarbonyl, benzyloxycarbonyl and the like. Lower-acyl refers to groups containing one to four carbons.

[0027] Aryl and heteroaryl mean a 5- or 6-membered aromatic or heteroaromatic ring containing 0-3 heteroatoms selected from O, N, or S; a bicyclic 9- or 10-membered aromatic or heteroaromatic ring system containing 0-3 heteroatoms selected from O, N,

or S; or a tricyclic 13- or 14-membered aromatic or heteroaromatic ring system containing 0-3 heteroatoms selected from O, N, or S. The aromatic 6- to 14-membered carbocyclic rings include, e.g., benzene, naphthalene, indane, tetralin, and fluorene and the 5- to 10-membered aromatic heterocyclic rings include, e.g., imidazole, pyridine, indole, thiophene, benzopyranone, thiazole, furan, benzimidazole, quinoline, isoquinoline, quinoxaline, pyrimidine, pyrazine, tetrazole and pyrazole.

[0028] Alkylaryl refers to a residue in which an aryl moiety is attached to the parent structure via an alkyl residue. Examples are benzyl, phenethyl, phenylvinyl, phenylallyl and the like. Alkylheteroaryl refers to a residue in which a heteroaryl moiety is attached to the parent structure via an alkyl residue. Examples include furanylmethyl, pyridinylmethyl, pyrimidinylethyl and the like.

[0029] Heterocycle means a cycloalkyl or aryl residue in which one to four of the carbons is replaced by a heteroatom such as oxygen, nitrogen or sulfur. Examples of heterocycles that fall within the scope of the invention include imidazoline, pyrrolidine, pyrazole, pyrrole, indole, quinoline, isoquinoline, tetrahydroisoquinoline, benzofuran, benzodioxan, benzodioxole (commonly referred to as methylenedioxyphenyl, when occurring as a substituent), tetrazole, morpholine, thiazole, pyridine, pyridazine, pyrimidine, thiophene, furan, oxazole, oxazoline, isoxazole, dioxane, tetrahydrofuran and the like. Examples of substituted heterocyclyl include 4-methyl-1-piperazinyl and 4-benzyl-1-piperidinyl.

[0030] Substituted alkyl, aryl and heteroaryl or heterocyclyl refer to alkyl, aryl, heteroaryl or heterocyclyl wherein H atoms are replaced with alkyl, halogen, hydroxy, alkoxy, alkylenedioxy (e.g. methylenedioxy) fluoroalkyl, carboxy (-COOH), carboalkoxy (i.e. acyloxy RCOO-), carboxyalkyl (-COOR), carboxamido, sulfonamidoalkyl, sulfonamidoaryl, aminocarbonyl, benzyloxycarbonylamino (CBZ-amino), cyano, carbonyl, nitro, dialkylamino, alkylamino, amino, alkylthio, alkylsulfinyl, alkylsulfonyl, alkylsulfonamido, arylthio, arylsulfinyl, arylsulfonyl, amidino, phenyl, benzyl, heteroaryl, heterocyclyl, phenoxy, benzyloxy, or heteroaryloxy. For the purposes of the present invention, substituted alkyl also includes oxaalkyl residues, i.e. alkyl residues in which one or more carbons has been replaced by oxygen.

[0031] Halogen refers to fluorine, chlorine, bromine or iodine. Fluorine, chlorine and bromine are preferred.

[0032] Many of the compounds described herein contain one or more asymmetric centers (e.g. the carbons to which R^2 and R^3 are attached) and may thus give rise to enantiomers, diastereomers, and other stereoisomeric forms that may be defined, in terms of absolute stereochemistry, as (R)- or (S)-. The present invention is meant to include all such possible isomers, including racemic mixtures, optically pure forms and intermediate mixtures. Optically active (R)- and (S)- isomers may be prepared using chiral synthons or chiral reagents, or resolved using conventional techniques. When the compounds described herein contain olefinic double bonds or other centers of geometric asymmetry, and unless specified otherwise, it is intended that the compounds include both E and Z geometric isomers. Likewise, all tautomeric forms are also intended to be included.

[0033] When desired, the R- and S-isomers may be resolved by methods known to those skilled in the art, for example by formation of diastereoisomeric salts or complexes which may be separated, for example, by crystallisation; via formation of diastereoisomeric derivatives which may be separated, for example, by crystallisation, gas-liquid or liquid chromatography; selective reaction of one enantiomer with an enantiomer-specific reagent, for example enzymatic oxidation or reduction, followed by separation of the modified and unmodified enantiomers; or gas-liquid or liquid chromatography in a chiral environment, for example on a chiral support, such as silica with a bound chiral ligand or in the presence of a chiral solvent. It will be appreciated that where the desired enantiomer is converted into another chemical entity by one of the separation procedures described above, a further step may be required to liberate the desired enantiomeric form. Alternatively, specific enantiomer may be synthesized by asymmetric synthesis using optically active reagents, substrates, catalysts or solvents, or by converting one enantiomer to the other by asymmetric transformation.

[0034] The graphic representations of racemic, ambiscalemic and scalemic or enantiomerically pure compounds used herein are taken from Maehr J. Chem. Ed. 62, 114-120 (1985): solid and broken wedges are used to denote the absolute configuration of a chiral element; wavy lines indicate disavowal of any stereochemical implication which the bond it represents could generate; solid and broken bold lines are geometric descriptors indicating the relative configuration shown but denoting racemic character; and wedge outlines and dotted or broken lines denote enantiomerically pure compounds of indeterminate absolute configuration.

[0035] In some embodiments, two R groups may be joined to form a ring structure. Again, the ring structure may contain heteroatoms and may be substituted with one or more substituents.

[0036] The compositions of the invention are synthesized as outlined below, utilizing techniques well known in the art. Once made, the compositions of the invention find use in a variety of applications. As will be appreciated by those in the art, mitosis may be altered in a variety of ways; that is, one can affect mitosis either by increasing or decreasing the activity of a component in the mitotic pathway. Stated differently, mitosis may be affected (e.g., disrupted) by disturbing equilibrium, either by inhibiting or activating certain components. Similar approaches may be used to alter meiosis.

[0037] In a preferred embodiment, the compositions of the invention are used to modulate mitotic spindle formation, thus causing prolonged cell cycle arrest in mitosis. By "modulate" herein is meant altering mitotic spindle formation, including increasing and decreasing spindle formation. By "mitotic spindle formation" herein is meant organization of microtubules into bipolar structures by mitotic kinesins. By "mitotic spindle dysfunction" herein is meant mitotic arrest and monopolar spindle formation.

[0038] The compositions of the invention are useful to bind to and/or modulate the activity of a mitotic kinesin, KSP. In a preferred embodiment, the KSP is human KSP, although KSP kinesins from other organisms may also be used. In this context, modulate means either increasing or decreasing spindle pole separation, causing malformation, i.e., splaying, of mitotic spindle poles, or otherwise causing morphological perturbation of the mitotic spindle. Also included within the definition of KSP for these purposes are variants and/or fragments of KSP. See U.S. Patent Application "Methods of Screening for Modulators of Cell Proliferation and Methods of Diagnosing Cell Proliferation States", filed Oct. 27, 1999 (U.S. Serial Number 09/428,156), hereby incorporated by reference in its entirety. In addition, other mitotic kinesins may be used in the present invention. However, the compositions of the invention have been shown to have specificity for KSP.

[0039] For assay of activity, generally either KSP or a compound according to the invention is non-diffusably bound to an insoluble support having isolated sample receiving areas (e.g., a microtiter plate, an array, etc.). The insoluble support may be made of any composition to which the compositions can be bound, is readily separated from soluble material, and is otherwise compatible with the overall method of screening.

The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports include microtiter plates, arrays, membranes and beads. These are typically made of glass, plastic (e.g., polystyrene), polysaccharides, nylon or nitrocellulose, Teflon™, etc. Microtiter plates and arrays are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples. The particular manner of binding of the composition is not crucial so long as it is compatible with the reagents and overall methods of the invention, maintains the activity of the composition and is nondiffusable. Preferred methods of binding include the use of antibodies (which do not sterically block either the ligand binding site or activation sequence when the protein is bound to the support), direct binding to "sticky" or ionic supports, chemical crosslinking, the synthesis of the protein or agent on the surface, etc. Following binding of the protein or agent, excess unbound material is removed by washing. The sample receiving areas may then be blocked through incubation with bovine serum albumin (BSA), casein or other innocuous protein or other moiety.

[0040] The antimitotic agents of the invention may be used on their own to modulate the activity of a mitotic kinesin, particularly KSP. In this embodiment, the mitotic agents of the invention are combined with KSP and the activity of KSP is assayed. Kinesin activity is known in the art and includes one or more kinesin activities. Kinesin activities include the ability to affect ATP hydrolysis; microtubule binding; gliding and polymerization/depolymerization (effects on microtubule dynamics); binding to other proteins of the spindle; binding to proteins involved in cell-cycle control; serving as a substrate to other enzymes; such as kinases or proteases; and specific kinesin cellular activities such as spindle pole separation.

[0041] Methods of performing motility assays are well known to those of skill in the art. (See e.g., Hall, et al. (1996), Biophys. J., 71: 3467-3476, Turner et al., 1996, Anal. Biochem. 242 (1):20-5; Gittes et al., 1996, Biophys. J. 70(l): 418-29; Shirakawa et al., 1995, J. Exp. BioL 198: 1809-15; Winkelmann et al., 1995, Biophys. J. 68: 2444-53; Winkelmann et al., 1995, Biophys. J. 68: 72S.)

[0042] Methods known in the art for determining ATPase hydrolysis activity also can be used. Preferably, solution based assays are utilized. U.S. application 09/314,464, filed May 18, 1999, hereby incorporated by reference in its entirety, describes such assays. Alternatively, conventional methods are used. For example, P_i

release from kinesin can be quantified. In one preferred embodiment, the ATPase hydrolysis activity assay utilizes 0.3 M PCA (perchloric acid) and malachite green reagent (8.27 mM sodium molybdate II, 0.33 mM malachite green oxalate, and 0.8 mM Triton X-1 00). To perform the assay, 10 μL of reaction is quenched in 90 μL of cold 0.3 M PCA. Phosphate standards are used so data can be converted to mM inorganic phosphate released. When all reactions and standards have been quenched in PCA, 100 μL of malachite green reagent is added to the relevant wells in e.g., a microtiter plate. The mixture is developed for 10-15 minutes and the plate is read at an absorbance of 650 nm. If phosphate standards were used, absorbance readings can be converted to mM P_i and plotted over time. Additionally, ATPase assays known in the art include the luciferase assay.

[0043] ATPase activity of kinesin motor domains also can be used to monitor the effects of modulating agents. In one embodiment ATPase assays of kinesin are performed in the absence of microtubules. In another embodiment, the ATPase assays are performed in the presence of microtubules. Different types of modulating agents can be detected in the above assays. In a preferred embodiment, the effect of a modulating agent is independent of the concentration of microtubules and ATP. In another embodiment, the effect of the agents on kinesin ATPase can be decreased by increasing the concentrations of ATP, microtubules or both. In yet another embodiment, the effect of the modulating agent is increased by increasing concentrations of ATP, microtubules or both.

[0044] Agents that modulate the biochemical activity of KSP in vitro may then be screened in vivo. Methods for such agents in vivo include assays of cell cycle distribution, cell viability, or the presence, morphology, activity, distribution, or amount of mitotic spindles. Methods for monitoring cell cycle distribution of a cell population, for example, by flow cytometry, are well known to those skilled in the art, as are methods for determining cell viability. See for example, U.S. Patent Application "Methods of Screening for Modulators of Cell Proliferation and Methods of Diagnosing Cell Proliferation States," filed Oct. 22, 1999, serial number 09/428,156, hereby incorporated by reference in its entirety.

[0045] In addition to the assays described above, microscopic methods for monitoring spindle formation and malformation are well known to those of skill in the

art (see, e.g., Whitehead and Rattner (1998), J. Cell Sci. 111:2551-61; Galgio et al, (1996) J. Cell biol., 135:399-414).

[0046] The compositions of the invention inhibit the KSP kinesin. One measure of inhibition is IC_{50}, defined as the concentration of the composition at which the activity of KSP is decreased by fifty percent. Preferred compositions have IC_{50}'s of less than about 1 mM, with preferred embodiments having IC_{50}'s of less than about 100 μM, with more preferred embodiments having IC_{50}'s of less than about 10 μM, with particularly preferred embodiments having IC_{50}'s of less than about 1 μM, and especially preferred embodiments having IC_{50}'s of less than about 500 nM. Measurement of IC_{50} is done using an ATPase assay.

[0047] Another measure of inhibition is K_i. For compounds with IC_{50}'s less than 1 μM, the K_i or K_d is defined as the dissociation rate constant for the interaction of the phenothiazine with KSP. Preferred compounds have K_i's of less than about 100 μM, with preferred embodiments having K_i's of less than about 10 μM, and particularly preferred embodiments having K_i's of less than about 1 μM and especially preferred embodiments having K_i's of less than about 500 nM.

[0048] Another measure of inhibition is GI_{50}, defined as the concentration of the compound that results in a decrease in the rate of cell growth by fifty percent. Preferred compounds have GI_{50}'s of less than about 1 mM. The level of preferability of embodiments is a function of their GI_{50} : those having GI_{50}'s of less than about 20 μM are more preferred; those having GI_{50}'s of 10 μM more so; those having GI_{50} of less than about 1 μM more so; those having GI_{50}'s of 500 nM more so. Measurement of GI_{50} is done using a cell proliferation assay.

[0049] The compositions of the invention are used to treat cellular proliferation diseases. Disease states which can be treated by the methods and compositions provided herein include, but are not limited to, cancer (further discussed below), autoimmune disease, arthritis, graft rejection, inflammatory bowel disease, proliferation induced after medical procedures, including, but not limited to, surgery, angioplasty, and the like. It is appreciated that in some cases the cells may not be in a hyper or hypo proliferation state (abnormal state) and still require treatment. For example, during wound healing, the cells may be proliferating "normally", but proliferation enhancement may be desired. Similarly, as discussed above, in the agriculture arena, cells may be in a "normal" state,

but proliferation modulation may be desired to enhance a crop by directly enhancing growth of a crop, or by inhibiting the growth of a plant or organism which adversely affects the crop. Thus, in one embodiment, the invention herein includes application to cells or individuals afflicted or impending affliction with any one of these disorders or states.

[0050] The compositions and methods provided herein are particularly deemed useful for the treatment of cancer including solid tumors such as skin, breast, brain, cervical carcinomas, testicular carcinomas, etc. More particularly, cancers that may be treated by the compositions and methods of the invention include, but are not limited to: Cardiac: sarcoma (angiosarcoma, fibrosarcoma, rhabdomyosarcoma, liposarcoma), myxoma, rhabdomyoma, fibroma, lipoma and teratoma; Lung: bronchogenic carcinoma (squamous cell, undifferentiated small cell, undifferentiated large cell, adenocarcinoma), alveolar (bronchiolar) carcinoma, bronchial adenoma, sarcoma, lymphoma, chondromatous hamartoma, mesothelioma; Gastrointestinal: esophagus (squamous cell carcinoma, adenocarcinoma, leiomyosarcoma, lymphoma), stomach (carcinoma, lymphoma, leiomyosarcoma), pancreas (ductal adenocarcinoma, insulinoma, glucagonoma, gastrinoma, carcinoid tumors, vipoma), small bowel (adenocarcinoma, lymphoma, carcinoid tumors, Karposi's sarcoma, leiomyoma, hemangioma, lipoma, neurofibroma, fibroma), large bowel (adenocarcinoma, tubular adenoma, villous adenoma, hamartoma, leiomyoma); Genitourinary tract: kidney (adenocarcinoma, Wilm's tumor (nephroblastoma), lymphoma, leukemia), bladder and urethra (squamous cell carcinoma, transitional cell carcinoma, adenocarcinoma), prostate (adenocarcinoma, sarcoma), testis (seminoma, teratoma, embryonal carcinoma, teratocarcinoma, choriocarcinoma, sarcoma, interstitial cell carcinoma, fibroma, fibroadenoma, adenomatoid tumors, lipoma); Liver: hepatoma (hepatocellular carcinoma), cholangiocarcinoma, hepatoblastoma, angiosarcoma, hepatocellular adenoma, hemangioma; Bone: osteogenic sarcoma (osteosarcoma), fibrosarcoma, malignant fibrous histiocytoma, chondrosarcoma, Ewing's sarcoma, malignant lymphoma (reticulum cell sarcoma), multiple myeloma, malignant giant cell tumor chordoma, osteochronfroma (osteocartilaginous exostoses), benign chondroma, chondroblastoma, chondromyxofibroma, osteoid osteoma and giant cell tumors; Nervous system: skull (osteoma, hemangioma, granuloma, xanthoma, osteitis deformans), meninges (meningioma, meningiosarcoma, gliomatosis), brain (astrocytoma, medulloblastoma,

glioma, ependymoma, germinoma (pinealoma), glioblastoma multiform, oligodendroglioma, schwannoma, retinoblastoma, congenital tumors), spinal cord neurofibroma, meningioma, glioma, sarcoma); Gynecological: uterus (endometrial carcinoma), cervix (cervical carcinoma, pre-tumor cervical dysplasia), ovaries (ovarian carcinoma (serous cystadenocarcinoma, mucinous cystadenocarcinoma, unclassified carcinoma), granulosa-thecal cell tumors, Sertoli-Leydig cell tumors, dysgerminoma, malignant teratoma), vulva (squamous cell carcinoma, intraepithelial carcinoma, adenocarcinoma, fibrosarcoma, melanoma), vagina (clear cell carcinoma, squamous cell carcinoma, botryoid sarcoma (embryonal rhabdomyosarcoma), fallopian tubes (carcinoma); Hematologic: blood (myeloid leukemia (acute and chronic), acute lymphoblastic leukemia, chronic lymphocytic leukemia, myeloproliferative diseases, multiple myeloma, myelodysplastic syndrome), Hodgkin's disease, non-Hodgkin's lymphoma (malignant lymphoma); Skin: malignant melanoma, basal cell carcinoma, squamous cell carcinoma, Karposi's sarcoma, moles dysplastic nevi, lipoma, angioma, dermatofibroma, keloids, psoriasis; and Adrenal glands: neuroblastoma. Thus, the term "cancerous cell" as provided herein, includes a cell afflicted by any one of the above identified conditions.

[0051] Accordingly, the compositions of the invention are administered to cells. By "administered" herein is meant administration of a therapeutically effective dose of the mitotic agents of the invention to a cell either in cell culture or in a patient. By "therapeutically effective dose" herein is meant a dose that produces the effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques. As is known in the art, adjustments for systemic versus localized delivery, age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the condition may be necessary, and will be ascertainable with routine experimentation by those skilled in the art. By "cells" herein is meant cells in which mitosis or meiosis can be altered.

[0052] A "patient" for the purposes of the present invention includes both humans and other animals, particularly mammals, and other organisms. Thus the methods are applicable to both human therapy and veterinary applications. In the preferred embodiment the patient is a mammal, and in the most preferred embodiment the patient is human.

[0053] Mitotic agents having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a patient, as described herein. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways as discussed below. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%. The agents may be administered alone or in combination with other treatments, i.e., radiation, or other chemotherapeutic agents.

[0054] In a preferred embodiment, the pharmaceutical compositions are in a water soluble form, such as pharmaceutically acceptable salts, which is meant to include both acid and base addition salts. "Pharmaceutically acceptable acid addition salt" refers to those salts that retain the biological effectiveness of the free bases and that are not biologically or otherwise undesirable, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like. "Pharmaceutically acceptable base addition salts" include those derived from inorganic bases such as sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum salts and the like. Particularly preferred are the ammonium, potassium, sodium, calcium, and magnesium salts. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, and ethanolamine.

[0055] The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents. The pharmaceutical compositions may also

include one or more of the following: carrier proteins such as serum albumin; buffers; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents; sweeteners and other flavoring agents; coloring agents; and polyethylene glycol. Additives are well known in the art, and are used in a variety of formulations.

[0056] The administration of the mitotic agents of the present invention can be done in a variety of ways as discussed above, including, but not limited to, orally, subcutaneously, intravenously, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, or intraocularly. In some instances, for example, in the treatment of wounds and inflammation, the anti-mitotic agents may be directly applied as a solution or spray.

[0057] To employ the compounds of the invention in a method of screening for compounds that bind to KSP kinesin, the KSP is bound to a support, and a compound of the invention (which is a mitotic agent) is added to the assay. Alternatively, the compound of the invention is bound to the support and KSP is added. Classes of compounds among which novel binding agents may be sought include specific antibodies, non-natural binding agents identified in screens of chemical libraries, peptide analogs, etc. Of particular interest are screening assays for candidate agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including labeled in vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, functional assays (phosphorylation assays, etc.) and the like.

[0058] The determination of the binding of the mitotic agent to KSP may be done in a number of ways. In a preferred embodiment, the mitotic agent (the compound of the invention) is labeled, for example, with a fluorescent or radioactive moiety and binding determined directly. For example, this may be done by attaching all or a portion of KSP to a solid support, adding a labeled mitotic agent (for example a compound of the invention in which at least one atom has been replaced by a detectable isotope), washing off excess reagent, and determining whether the amount of the label is that present on the solid support. Various blocking and washing steps may be utilized as is known in the art.

[0059] By "labeled" herein is meant that the compound is either directly or indirectly labeled with a label which provides a detectable signal, e.g., radioisotope, fluorescent tag, enzyme, antibodies, particles such as magnetic particles,

chemiluminescent tag, or specific binding molecules, etc. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule which provides for detection, in accordance with known procedures, as outlined above. The label can directly or indirectly provide a detectable signal.

[0060] In some embodiments, only one of the components is labeled. For example, the kinesin proteins may be labeled at tyrosine positions using ^{125}I, or with fluorophores. Alternatively, more than one component may be labeled with different labels; using ^{125}I for the proteins, for example, and a fluorophor for the mitotic agents.

[0061] The compounds of the invention may also be used as competitors to screen for additional drug candidates. "Candidate bioactive agent" or "drug candidate" or grammatical equivalents as used herein describe any molecule, e.g., protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, etc., to be tested for bioactivity. They may be capable of directly or indirectly altering the cellular proliferation phenotype or the expression of a cellular proliferation sequence, including both nucleic acid sequences and protein sequences. In other cases, alteration of cellular proliferation protein binding and/or activity is screened. Screens of this sort may be performed either in the presence or absence of microtubules. In the case where protein binding or activity is screened, preferred embodiments exclude molecules already known to bind to that particular protein, for example, polymer structures such as microtubules, and energy sources such as ATP. Preferred embodiments of assays herein include candidate agents which do not bind the cellular proliferation protein in its endogenous native state termed herein as "exogenous" agents. In another preferred embodiment, exogenous agents further exclude antibodies to KSP.

[0062] Candidate agents can encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding and lipophilic binding, and typically include at least an amine, carbonyl, hydroxyl, ether, or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including

peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are peptides.

[0063] Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

[0064] Competitive screening assays may be done by combining KSP and a drug candidate in a first sample. A second sample comprises a mitotic agent, KSP and a drug candidate. This may be performed in either the presence or absence of microtubules. The binding of the drug candidate is determined for both samples, and a change, or difference in binding between the two samples indicates the presence of an agent capable of binding to KSP and potentially modulating its activity. That is, if the binding of the drug candidate is different in the second sample relative to the first sample, the drug candidate is capable of binding to KSP.

[0065] In a preferred embodiment, the binding of the candidate agent is determined through the use of competitive binding assays. In this embodiment, the competitor is a binding moiety known to bind to KSP, such as an antibody, peptide, binding partner, ligand, etc. Under certain circumstances, there may be competitive binding as between the candidate agent and the binding moiety, with the binding moiety displacing the candidate agent.

[0066] In one embodiment, the candidate agent is labeled. Either the candidate agent, or the competitor, or both, is added first to KSP for a time sufficient to allow binding, if present. Incubations may be performed at any temperature which facilitates optimal activity, typically between 4 and 40°C.

[0067] Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high throughput screening. Typically between 0.1 and 1 hour will be sufficient. Excess reagent is generally removed or washed away. The

second component is then added, and the presence or absence of the labeled component is followed, to indicate binding.

[0068] In a preferred embodiment, the competitor is added first, followed by the candidate agent. Displacement of the competitor is an indication the candidate agent is binding to KSP and thus is capable of binding to, and potentially modulating, the activity of KSP. In this embodiment, either component can be labeled. Thus, for example, if the competitor is labeled, the presence of label in the wash solution indicates displacement by the agent. Alternatively, if the candidate agent is labeled, the presence of the label on the support indicates displacement.

[0069] In an alternative embodiment, the candidate agent is added first, with incubation and washing, followed by the competitor. The absence of binding by the competitor may indicate the candidate agent is bound to KSP with a higher affinity. Thus, if the candidate agent is labeled, the presence of the label on the support, coupled with a lack of competitor binding, may indicate the candidate agent is capable of binding to KSP.

[0070] It may be of value to identify the binding site of KSP. This can be done in a variety of ways. In one embodiment, once KSP has been identified as binding to the mitotic agent, KSP is fragmented or modified and the assays repeated to identify the necessary components for binding.

[0071] Modulation is tested by screening for candidate agents capable of modulating the activity of KSP comprising the steps of combining a candidate agent with KSP, as above, and determining an alteration in the biological activity of KSP. Thus, in this embodiment, the candidate agent should both bind to KSP (although this may not be necessary), and alter its biological or biochemical activity as defined herein. The methods include both in vitro screening methods and in vivo screening of cells for alterations in cell cycle distribution, cell viability, or for the presence, morpohology, activity, distribution, or amount of mitotic spindles, as are generally outlined above.

[0072] Alternatively, differential screening may be used to identify drug candidates that bind to the native KSP, but cannot bind to modified KSP.

[0073] Positive controls and negative controls may be used in the assays. Preferably all control and test samples are performed in at least triplicate to obtain statistically significant results. Incubation of all samples is for a time sufficient for the binding of the agent to the protein. Following incubation, all samples are washed free of

non-specifically bound material and the amount of bound, generally labeled agent determined. For example, where a radiolabel is employed, the samples may be counted in a scintillation counter to determine the amount of bound compound.

[0074] A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g., albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in any order that provides for the requisite binding.

[0075] The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference in their entirety.

EXAMPLES

Abbreviations and Definitions

[0076] The following abbreviations and terms have the indicated meanings throughout:

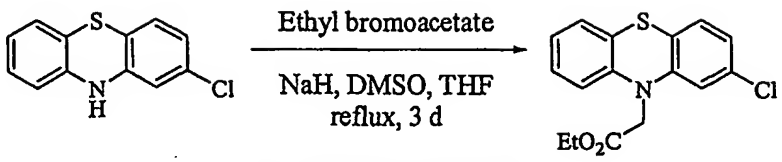
Ac = acetyl
BNB = 4-bromomethyl-3-nitrobenzoic acid
Boc = t-butyloxy carbonyl
Bu = butyl
c- = cyclo
CBZ = carbobenzoxy = benzyloxycarbonyl
DBU = diazabicyclo[5.4.0]undec-7-ene
DCM = dichloromethane = methylene chloride = CH_2Cl_2
DCE = dichloroethane
DEAD = diethyl azodicarboxylate
DIC = diisopropylcarbodiimide
DIEA = N,N-diisopropylethylamine
DMAP = 4-N,N-dimethylaminopyridine
DMF = N,N-dimethylformamide
DMSO = dimethyl sulfoxide
DVB = 1,4-divinylbenzene
EDCI = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EEDQ = 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
Et = ethyl
Fmoc = 9-fluorenylmethoxycarbonyl
GC = gas chromatography
HATU = O-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HMDS = hexamethyldisilazane
HOAc = acetic acid
HOBt = hydroxybenzotriazole
Me = methyl
mesyl = methanesulfonyl

MTBE = methyl t-butyl ether
NMO = N-methylmorpholine oxide
PEG = polyethylene glycol
Ph = phenyl
PhOH = phenol
PfP = pentafluorophenol
PPTS = pyridinium p-toluenesulfonate
Py = pyridine
PyBroP = bromo-tris-pyrrolidino-phosphonium hexafluorophosphate
rt = room temperature
sat=d = saturated
s- = secondary
t- = tertiary
TBDMS = t-butyldimethylsilyl
TES = triethylsilane
TFA = trifluoroacetic acid
THF = tetrahydrofuran
TMOF = trimethyl orthoformate
TMS = trimethylsilyl
tosyl = p-toluenesulfonyl
Trt = triphenylmethyl

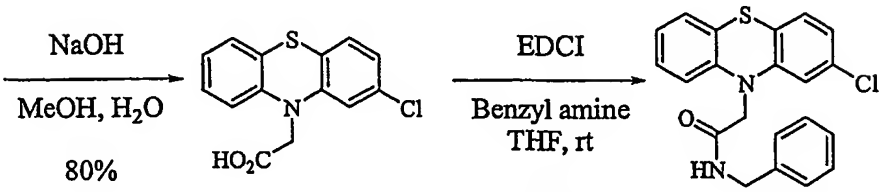
Synthesis of Compounds

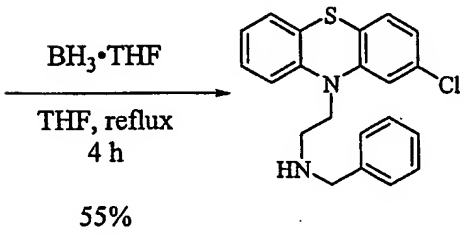
[0077] The syntheses of several prototypical phenothiazines are shown below. Other phenothiazines are made in analogous fashion:

Phenothiazine Synthesis - Procedure A

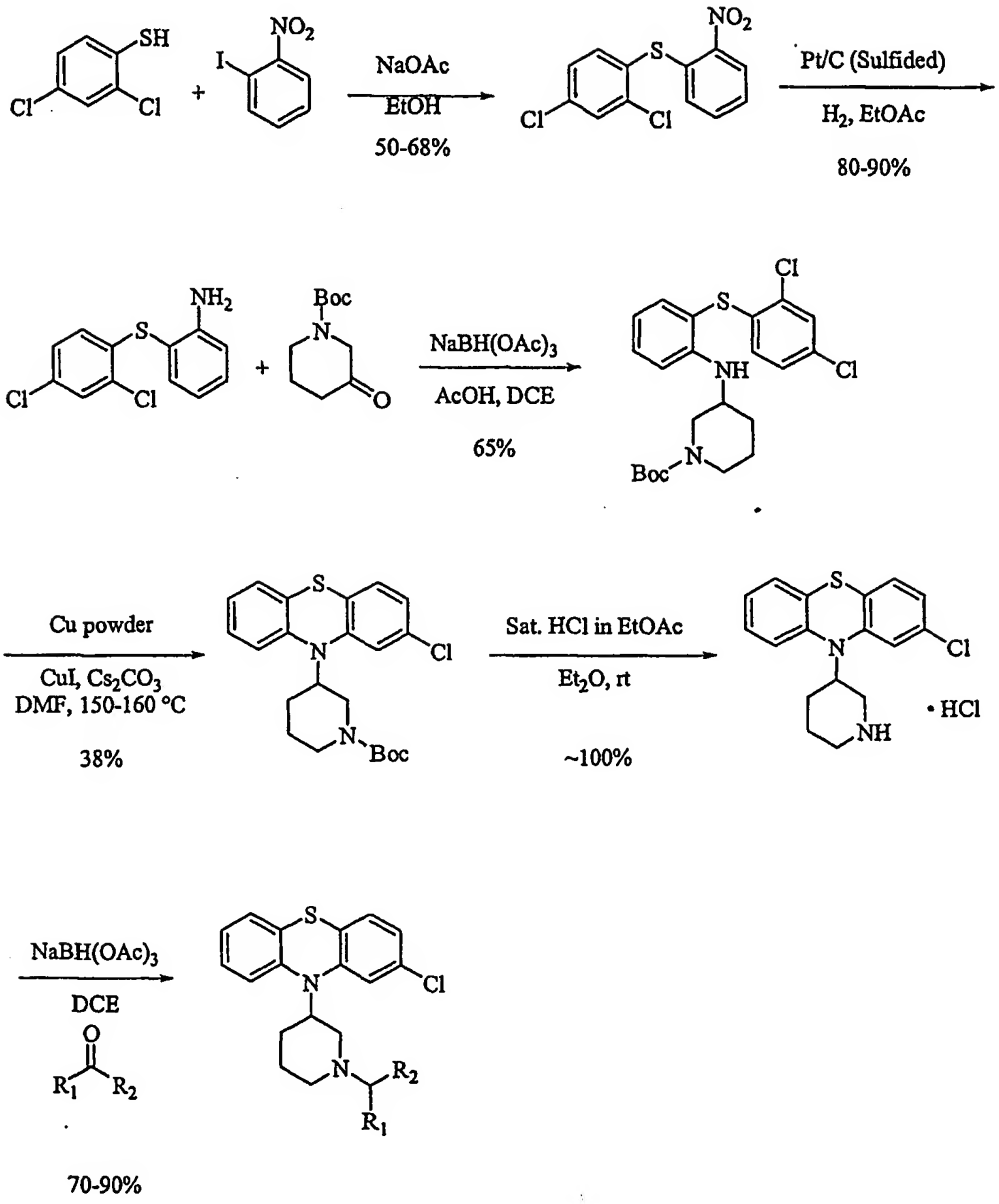


quantitative crude yield



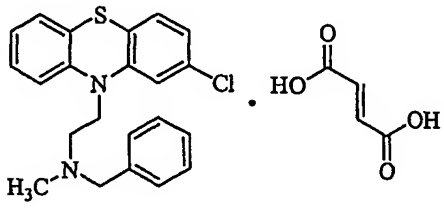


Phenothiazine Synthesis - Procedure B



Phenothiazine Procedure A

[0078] Synthesis of Benzyl-[2-(2-chloro-phenothiazin-10-yl)-ethyl]-methylamine fumarate.



Synthesis of (2-chlorophenothiazin-10-yl)acetic acid.

[0079] 2-Chloro-10*H*-phenothiazine (10.0 g, 42.8 mmol) was dissolved in THF (100 mL) and DMSO (10 mL). Sodium hydride (1.0 g, 43.4 mmol) was added, and the mixture was heated to reflux until gas evolution ceased. Ethyl bromoacetate (10 g, 59.9 mmol) was added slowly via syringe. The mixture was heated at reflux for 12 h. Sodium hydride (1.0 g, 43.4 mmol) and ethyl bromoacetate (5 g, 29.9 mmol) were added, and the mixture was heated an additional 12 h. The mixture was cooled to room temperature and carefully diluted with water followed by ethyl acetate. The layers were separated, and the aqueous layer extracted with ethyl acetate. The organic layers were combined, dried (Na_2SO_4), filtered, and concentrated to give a dark purple oil (11.8 g). The oil was dissolved in methanol (300 mL), and aqueous sodium hydroxide (6 N, 22 mL, 132 mmol) was added. The mixture was heated to reflux for 4 h. The mixture was cooled to room temperature, and the solvent removed under vacuum. The residue was dissolved in water and was extracted with diethyl ether (ether layers discarded). The layers were separated, and the pH of the aqueous layer was made acidic with concentrated hydrochloric acid. The water layer was extracted with methylene chloride. The organic layers were combined, dried (Na_2SO_4), filtered, and concentrated to give (2-chlorophenothiazin-10-yl)acetic acid (7.38 g, 56%) as a brown solid.

Synthesis of *N*-Benzyl-2-(2-chlorophenothiazin-10-yl)-*N*-methylacetamide

[0080] (2-Chlorophenothiazin-10-yl)acetic acid (480 mg, 1.65 mmol) was dissolved in THF (10 mL). To this solution, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.58 g, 8.25 mmol), 1-hydroxybenzotriazole (220 mg,

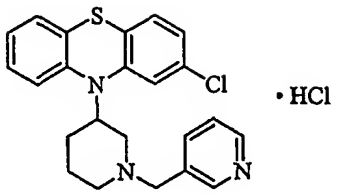
1.65 mmol), and *N*-methyl benzyl amine (2.0 mL, 16.5 mmol) were added. The mixture was stirred at room temperature overnight and was then diluted with water and methylene chloride. The aqueous layer was made basic with sodium hydroxide (1N), and the layers were separated. The aqueous layer was extracted with methylene chloride. The organic layers were combined, washed with water and brine, dried (Na_2SO_4), filtered, and concentrated to provide a mixture of *N*-methyl benzyl amine and product (3.8 g). The residue was purified with a plug of SiO_2. The product was recrystallized from hexane and ethyl acetate to provide *N*-benzyl-2-(2-chlorophenothiazin-10-yl)-*N*-methylacetamide as a white solid (258 mg, 40%).

Synthesis of Benzyl-[2-(2-chlorophenothiazin-10-yl)-ethyl]methyl amine fumarate.

[0081] *N*-Benzyl-2-(2-chlorophenothiazin-10-yl)-*N*-methylacetamide (140 mg, 0.355 mmol) was dissolved in THF (5 mL). A solution of borane • THF complex in THF (1.0 M, 5 mL, 5 mmol) was added, and the solution heated to reflux for 4 h. The mixture was carefully diluted with saturated HCl in methanol and stirred for 30 min. The solvent was removed under vacuum, and the residue dissolved in ethyl acetate and aqueous sodium hydroxide (1 N). The layers were separated and the aqueous layer extracted with ethyl acetate. The organic layers were combined, dried (Na_2SO_4), filtered, and concentrated to provide benzyl-[2-(2-chlorophenothiazin-10-yl)-ethyl]methyl amine as a white solid (160 mg). The crude product was purified by SiO_2 chromatography to provide pure product (50 mg, 34%) and 100 mg of impure product. The pure product was dissolved in acetone and a solution of fumaric acid (0.8 mL, 0.02 g/mL in methanol) was added. The solvents were removed under vacuum, and the residue slurried in chloroform and filtered to provide the sub-titled compound (47 mg) as an off-white solid.

Phenothiazine Procedure B

Synthesis of 2-Chloro-10-(1-pyridin-3-ylmethylpiperidin-3-yl)-10*H*-phenothiazine hydrochloride.



Synthesis of 2,4-dichloro-2'-nitrodiphenyl thioether.

[0082] 2,4-Dichlorobenzenethiol (16.1 g, 89.9 mmol) was dissolved in ethanol (200 mL) and was heated to reflux. A solution of sodium acetate (11.1 g, 135 mmol) dissolved in 100 mL ethanol was added, followed by a solution of 1-iodo-2-nitrobenzene (33.6 g, 135 mmol) in ethanol (100 mL, added in small portions over 5 min). The mixture was heated at reflux for 12 h. The mixture was cooled to room temperature and concentrated under vacuum. The residue was dissolved in water and methylene chloride, and the aqueous layer was diluted with saturated sodium bicarbonate. The layers were separated, and the aqueous layer was extracted with methylene chloride. The organic layers were combined, dried (Na_2SO_4), filtered, and concentrated to provide a solid. The residue was washed with 30% ethanol in water (300 mL) and collected by vacuum filtration. The filter cake was rinsed with 30% ethanol in water. The solid was treated with methanol (100 mL), and the product collected by filtration to provide 2,4-dichloro-2'-nitrodiphenyl thioether (25.6 g, 94%) as a yellow solid.

Synthesis of 2,4-dichloro-2'-aminodiphenyl thioether

[0083] 2,4-Dichloro-2'-nitrodiphenyl thioether (22.4 g, 75 mmol) was dissolved in ethyl acetate (125 mL) at 40 °C. Adams catalyst (PtO_2, 2.5 g) was added, and hydrogen was vigorously bubbled through the solution for 1 h. The mixture was stirred overnight under a static atmosphere of hydrogen. The mixture was cooled to room temperature and filtered through a bed of cellulose. The solvent was removed under vacuum to provide 2,4-dichloro-2'-aminodiphenyl thioether (18.1 g, 89%) as a yellow oil.

Synthesis of 3-Hydroxypiperidine-1-carboxylic acid *tert*-butyl ester

[0084] 3-Hydroxypiperidine (10.0 g, 98.9 mmol) was dissolved in methylene chloride (100 mL) and cooled to 0 °C. Di-*tert*-butyl dicarbonate (27.3 g, 125 mmol) was added at once (copious gas evolution was observed). The mixture was stirred for 12 h at room temperature. The solvent was removed under vacuum to provide 3-hydroxypiperidine-1-carboxylic acid *tert*-butyl ester (19.8 g, 100%).

Synthesis of 3-Oxopiperidine-1-carboxylic acid *tert*-butyl ester.

[0085] A mixture of DMSO (7.8 mL, 109.3 mmol) and methylene chloride (100 mL) was cooled to –78 °C. Oxalyl chloride (4.8 mL, 54.7 mmol) was added to this solution via syringe. The mixture was stirred at –78 °C for 30 min. A solution of 3-hydroxypiperidine-1-carboxylic acid *tert*-butyl ester (10.0 g, 49.7 mmol) in methylene chloride (30 mL) was added dropwise to this solution (temperature remained below –70 °C). The mixture was stirred for 30 min. Triethylamine (28 mL, 200 mmol) was added dropwise over 20 min. The mixture was stirred at –78 °C for 1 h and was allowed to warm to room temperature and stirred for 45 min. The mixture was diluted with water, and the layers separated. The organic layer was washed with water, dried (Na_2SO_4), filtered, and concentrated to give 3-oxopiperidine-1-carboxylic acid *tert*-butyl ester (10.2 g) as a yellow, brown liquid.

Synthesis of 3-[2-(2,4-Dichlorophenylsulfanyl)phenylamino]piperidine-1-carboxylic acid *tert*-butyl ester.

[0086] 2,4-Dichloro-2'-aminodiphenyl thioether (10.3 g, 38.1 mmol) was dissolved in dichloroethane (120 mL). To this solution, a solution of 3-oxopiperidine-1-carboxylic acid *tert*-butyl ester (14.0 g, 70.3 mmol) in dichloroethane (20 mL) and solid sodium triacetoxyborohydride (14.5 g, 68.7 mmol) were slowly added. Acetic acid (5.4 mL, 94 mmol) was added slowly via syringe, and the mixture stirred for 48 h. The mixture was carefully diluted with water, and the pH was adjusted to approximately 9 with aqueous sodium hydroxide (1 N). The mixture was diluted with ethyl acetate, and the layers were separated. The aqueous layer was extracted with ethyl acetate. The organic layers were combined, dried (Na_2SO_4), filtered, and concentrated to provide 3-[2-(2,4-dichlorophenylsulfanyl)phenylamino]piperidine-1-carboxylic acid *tert*-butyl ester

as a brown oil. The crude product was purified by SiO_2 chromatography resulting in pure product (8.4 g, 49%) as a light yellow oil.

Synthesis of 3-(2-Chlorophenothiazin-10-yl)piperidine-1-carboxylic acid *tert*-butyl ester.

[0087] 3-[2-(2,4-Dichlorophenylsulfanyl)phenylamino]piperidine-1-carboxylic acid *tert*-butyl ester (8.4 g, 18.5 mmol) was dissolved in DMF (120 mL) and vigorously degassed with N_2 for 20 min. Cesium carbonate (27.2 g, 83.5 mmol), copper(I) iodide (5.28 g, 27.8 mmol), and copper powder (8.4 g, 132 mmol) were added to the solution. The suspension was stirred vigorously and heated to 155-156 °C for 12 hours (N_2 was continuously bubbled through the mixture). The mixture was cooled to room temperature and diluted with ethyl acetate. The solids were removed by vacuum filtration and were rinsed with ethyl acetate. The filtrate was concentrated under vacuum to remove 90% of the volatiles. The residue was purified by SiO_2 chromatography to provide 3-(2-chlorophenothiazin-10-yl) piperidine-1-carboxylic acid *tert*-butyl ester (4.1 g, 53%) as a white solid.

Synthesis of 2-Chloro-10-piperidin-3-yl-10*H*-phenothiazine hydrochloride

[0088] 3-(2-Chlorophenothiazin-10-yl) piperidine-1-carboxylic acid *tert*-butyl ester (4.1 g, 9.83 mmol) was dissolved in diethyl ether (200 mL), and the solution cooled to 0 °C. A solution of HCl in ethyl acetate (~4 M, 30 mL) was slowly added. The mixture was allowed to warm to room temperature and stirred for 8 h. The solvent was removed under vacuum. The residue treated with HCl in ethyl acetate (~4 M, 75 mL) and stirred overnight. The solvent was removed under vacuum to provide 2-chloro-10-piperidin-3-yl-10*H*-phenothiazine (3.98 g, 100%) as an off-white solid.

Synthesis of 2-Chloro-10-(1-pyridin-3-yl-methylpiperidin-3-yl)-10*H*-phenothiazine hydrochloride.

[0089] Using methods substantially equivalent to those described in the synthesis of 3-[2-(2,4-dichloro-phenylsulfanyl)-phenylamino]-piperidine-1-carboxylic acid *tert*-butyl ester,

[0090] 2-chloro-10-(1-pyridin-3-yl-methylpiperidin-3-yl)-10*H*-phenothiazine was prepared by treatment of 2-chloro-10-piperidin-3-yl-10*H*-phenothiazine (240 mg, 0.757 mmol) and 3-pyridinecarboxaldehyde (81 mg, 0.757 mmol) with sodium triacetoxyborohydride (224 mg, 1.06 mmol) to provide after treatment with ethereal HCl, the sub-titled compound (136 mg, 41%).

Induction of Mitotic Arrest in Cell Populations Treated with a Phenothiazine KSP Inhibitor

[0091] FACS analysis to determine cell cycle stage by measuring DNA content was performed as follows. Skov-3 cells (human ovarian cancer) were split 1:10 for plating in 10cm dishes and grown to subconfluence with RPMI 1640 medium containing 5% fetal bovine serum (FBS). The cells were then treated with either 10nM paclitaxel, the test compound or 0.25% DMSO (vehicle for compounds) for 24 hours. Cells were then rinsed off the plates with PBS containing 5mM EDTA, pelleted, washed once in PBS containing 1% FCS, and then fixed overnight in 85% ethanol at 4°C. Before analysis, the cells were pelleted, washed once, and stained in a solution of 10μg propidium iodide and 250μg of ribonuclease (RNAse) A per milliliter at 37°C for half an hour. Flow cytometry analysis was performed on a Becton-Dickinson FACScan, and data from 10,000 cells per sample was analyzed with Modfit software.

[0092] The phenothiazine compounds, as well as the known anti-mitotic agent paclitaxel, caused a shift in the population of cells from a G0/G1 cell cycle stage (2n DNA content) to a G2/M cell cycle stage (4n DNA content). Other compounds of this class were found to have similar effects.

Monopolar Spindle Formation following Application of a Phenothiazine KSP Inhibitor

[0093] To determine the nature of the G2/M accumulation, human tumor cell lines Skov-3 (ovarian), HeLa (cervical), and A549 (lung) were plated in 96-well plates at densities of 4,000 cells per well (SKOV-3 & HeLa) or 8,000 cells per well (A549),

allowed to adhere for 24 hours, and treated with various concentrations of the phenothiazine compounds for 24 hours. Cells were fixed in 4% formaldehyde and stained with antitubulin antibodies (subsequently recognized using fluorescently-labeled secondary antibody) and Hoechst dye (which stains DNA).

[0094] Visual inspection revealed that the phenothiazine compounds caused cell cycle arrest in the prometaphase stage of mitosis. DNA was condensed and spindle formation had initiated, but arrested cells uniformly displayed monopolar spindles, indicating that there was an inhibition of spindle pole body separation. Microinjection of anti-KSP antibodies also causes mitotic arrest with arrested cells displaying monopolar spindles.

Inhibition of Cellular Proliferation in Tumor Cell Lines Treated with Phenothiazine KSP Inhibitors.

[0095] Cells were plated in 96-well plates at densities from 1000-2500 cells/well of a 96-well plate (depending on the cell line) and allowed to adhere/grow for 24 hours. They were then treated with various concentrations of drug for 48 hours. The time at which compounds are added is considered T_0. A tetrazolium-based assay using the reagent 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (I.S> Patent No. 5,185,450) (see Promega product catalog #G3580, CellTiter 96® AQ_{ucous} One Solution Cell Proliferation Assay) was used to determine the number of viable cells at T_0 and the number of cells remaining after 48 hours compound exposure. The number of cells remaining after 48 hours was compared to the number of viable cells at the time of drug addition, allowing for calculation of growth inhibition.

[0096] The growth over 48 hours of cells in control wells that had been treated with vehicle only (0.25% DMSO) is considered 100% growth and the growth of cells in wells with compounds is compared to this. Phenothiazine KSP inhibitors inhibited cell proliferation in human tumor cell lines of the following tumor types: lung (NCI-H460, A549), breast (MDA-MB-231, MCF-7, MCF-7/ADR-RES), colon (HT29, HCT15), ovarian (SKOV-3, OVCAR-3), leukemia (HL-60(TB), K-562), central nervous system (SF-268), renal (A498), osteosarcoma (U2-OS), and cervical (HeLa). In addition, a mouse tumor line (B16, melanoma) was also growth-inhibited in the presence of the phenothiazine compounds.

[0097] A Gi_{50} was calculated by plotting the concentration of compound in μM vs the percentage of cell growth of cell growth in treated wells. The Gi_{50} calculated for the compounds is the estimated concentration at which growth is inhibited by 50% compared to control, i.e., the concentration at which:

$$100 \times [(Treated_{48} - T_0) / (Control_{48} - T_0)] = 50.$$

[0098] All concentrations of compounds are tested in duplicate and controls are averaged over 12 wells. A very similar 96-well plate layout and Gi_{50} calculation scheme is used by the National Cancer Institute (see Monks, et al., J. NatI. Cancer Inst. 83:757-766 (1991)). However, the method by which the National Cancer Institute quantitates cell number does not use MTS, but instead employs alternative methods.

Calculation Of IC_{50}:

[0099] Measurement of a composition's IC_{50} for KSP activity uses an ATPase assay. The following solutions are used: Solution 1 consists of 3 mM phosphoenolpyruvate potassium salt (Sigma P-7127), 2 mM ATP (Sigma A-3377), 1 mM IDTT (Sigma D-9779), 5 μM paclitaxel (Sigma T-7402), 10 ppm antifoam 289 (Sigma A-8436), 25 mM Pipes/KOH pH 6.8 (Sigma P6757), 2 mM MgC12 (VWR JT400301), and 1 mM EGTA (Sigma E3889). Solution 2 consists of 1 mM NADH (Sigma N8129), 0.2 mg/ml BSA (Sigma A7906), pyruvate kinase 7U/ml, L-lactate dehydrogenase 10 U/ml (Sigma P0294), 100 nM KSP motor domain, 50 μg/ml microtubules, 1 mM DTT (Sigma D9779), 5 μM paclitaxel (Sigma T-7402), 10 ppm antifoam 289 (Sigma A-8436), 25 mM Pipes/KOH pH 6.8 (Sigma P6757), 2 mM MgC12 (VWR JT4003-01), and 1 mM EGTA (Sigma E3889). Serial dilutions (8-12 two-fold dilutions) of the composition are made in a 96-well microtiter plate (Corning Costar 3695) using Solution 1. Following serial dilution each well has 50 μl of Solution 1. The reaction is started by adding 50 μl of solution 2 to each well. This may be done with a multichannel pipettor either manually or with automated liquid handling devices. The microtiter plate is then transferred to a microplate absorbance reader and multiple absorbance readings at 340 nm are taken for each well in a kinetic mode. The observed rate of change, which is proportional to the ATPase rate, is then plotted as a function of the compound concentration. For a standard IC_{50} determination the data acquired is fit by the following four parameter equation using a nonlinear fitting program (e.g., Grafit 4):

$$y = \frac{\text{Range}}{1 + \left(\frac{x}{IC_{50}}\right)^{s}} + \text{Background}$$

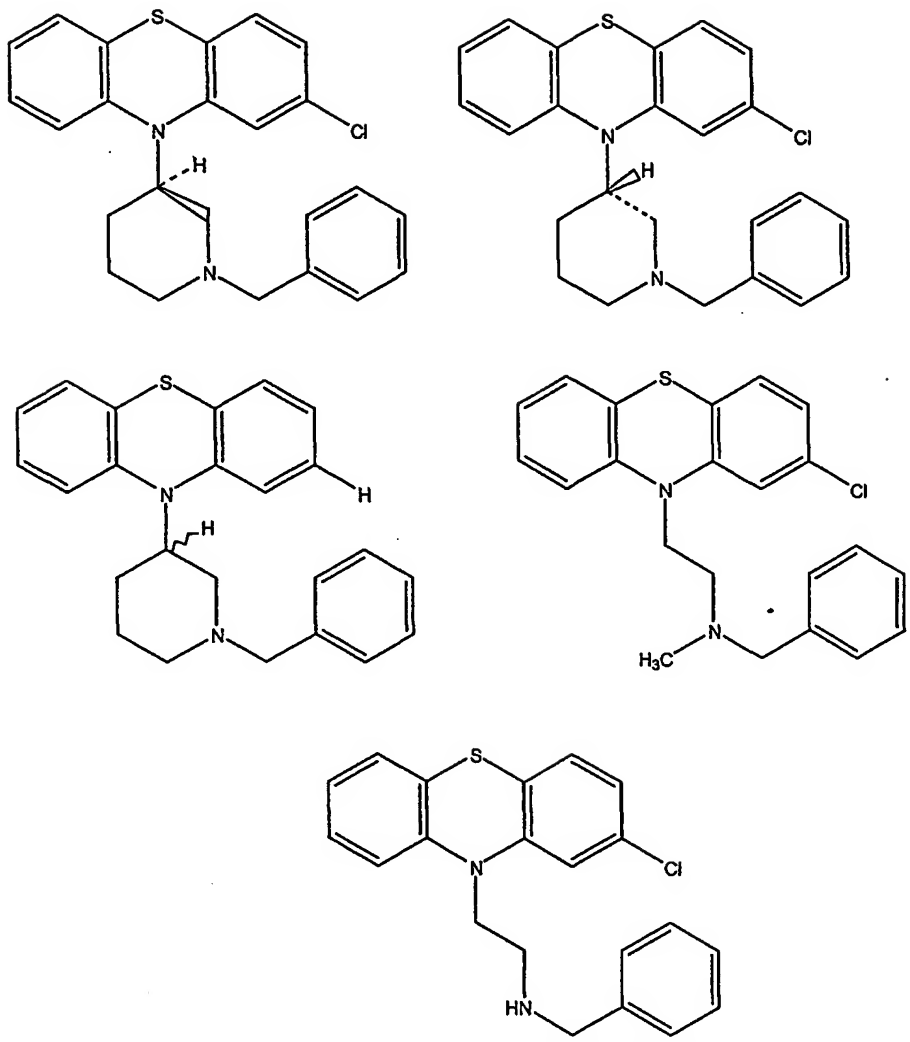
where y is the observed rate and x the compound concentration.

[00100] The K_i for a compound is determined from the IC_{50} based on three assumptions. First, only one compound molecule binds to the enzyme and there is no cooperativity. Second, the concentrations of active enzyme and the compound tested are known (i.e., there are no significant amounts of impurities or inactive forms in the preparations). Third, the enzymatic rate of the enzyme-inhibitor complex is zero. The rate (i.e., compound concentration) data are fitted to the equation:

$$V = V_{max}E_0 \left[I - \frac{(E_0 + I_0 + Kd) - \sqrt{(E_0 + I_0 + Kd)^2 - 4E_0I_0}}{2E_0} \right]$$

where V is the observed rate, V_{max} is the rate of the free enzyme, I_0 is the inhibitor concentration, E_0 is the enzyme concentration, and K_d is the dissociation constant of the enzyme-inhibitor complex.

[00101] Several representative compounds of the invention (as their fumarate salts) were tested as described above and found to exhibit Ki's less than 10 μM. Their structures are as shown:



[00102] The phenothiazine compounds inhibit growth in a variety of cell lines, including cell lines (MCF-7/ADR-RES, HCT1 5) that express P-glycoprotein (also known as Multi-drug Resistance, or MDR^+), which conveys resistance to other chemotherapeutic drugs, such as pacilitaxel. Therefore, the phenothiazines are anti-mitotics that inhibit cell proliferation, and are not subject to resistance by overexpression of MDR^+ by drug-resistant tumor lines.

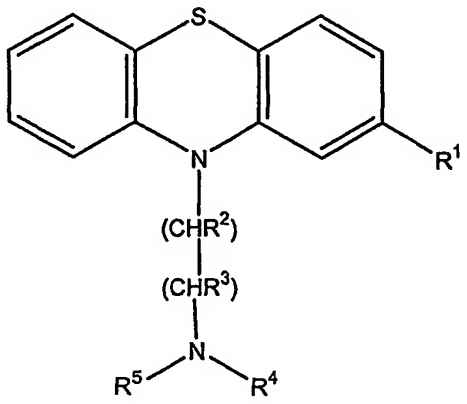
[00103] Compounds of this class were found to inhibit cell proliferation, although GI_{50} values varied. GI_{50} values for the phenothiazine compounds tested ranged from 200 nM to greater than the highest concentration tested. By this we mean that although most of the compounds that inhibited KSP activity biochemically did inhibit cell proliferation, for some, at the highest concentration tested (generally about 20 μM), cell growth was inhibited less than 50%. Many of the compounds have GI_{50} values less than 10 μM, and

several have GI_{50} values less than 1 μM. Anti-proliferative compounds that have been successfully applied in the clinic to treatment of cancer (cancer chemotherapeutics) have GI_{50}'s that vary greatly. For example, in A549 cells, paclitaxel GI_{50} is 4 nM, doxorubicin is 63 nM, 5-fluorouracil is 1 μM, and hydroxyurea is 500 μM (data provided by National Cancer Institute, Developmental Therapeutic Program, http://dtp.nci.nih.gov/). Therefore, compounds that inhibit cellular proliferation at virtually any concentration may be useful. However, preferably, compounds will have GI_{50} values of less than 1 mM. More preferably, compounds will have GI_{50} values of less than 20 μM. Even more preferably, compounds will have GI_{50} values of less than 10 μM. Further reduction in GI_{50} values may also be desirable, including compounds with GI_{50} values of less than 1 μM.

CLAIMS

We claim:

1. A method of treating cellular proliferative diseases comprising administering a compound of the formula



wherein

R^1 is hydrogen, halogen or CF_3;

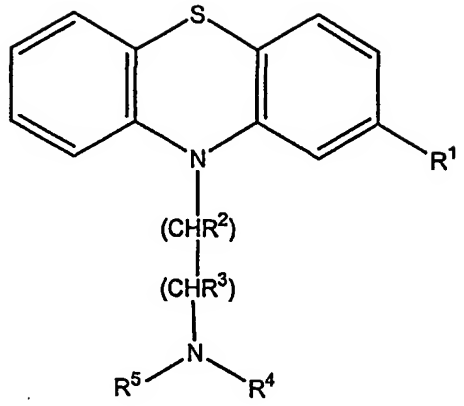
R^2 is chosen from hydrogen and lower alkyl;

R^3 is hydrogen;

R^4 and R^5 are independently chosen from hydrogen, alkyl, substituted alkyl, alkylaryl, substituted alkylaryl, alkylheteroaryl and substituted alkylheteroaryl; or

any of R^2, R^3 and R^4 taken together with the intervening atoms form one or more five- to seven-membered rings, said ring optionally substituted with one or more alkyl, aryl, alkoxy, halo, alkylaryl or substituted alkylaryl substituents, with the proviso that said phenothiazine must contain at least one additional five- to seven-membered ring, or a pharmaceutically acceptable salt thereof.

2. A method of treating a disorder associated with KSP kinesin activity comprising administering a compound of formula



wherein

R^1 is hydrogen, halogen or CF_3;

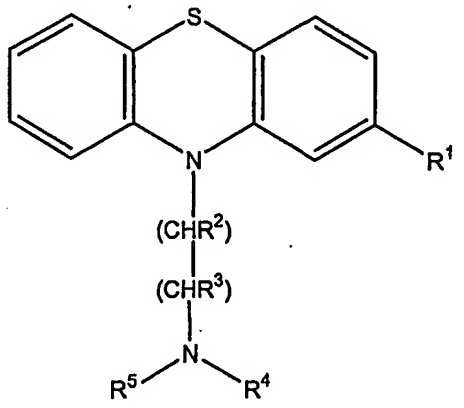
R^2 is chosen from hydrogen and lower alkyl;

R^3 is hydrogen;

R^4 and R^5 are independently chosen from hydrogen, alkyl, substituted alkyl, alkylaryl, substituted alkylaryl, alkylheteroaryl and substituted alkylheteroaryl; or

any of R^2, R^3 and R^4 taken together with the intervening atoms form one or more five- to seven-membered rings, said ring optionally substituted with one or more alkyl, aryl, alkoxy, halo, alkylaryl or substituted alkylaryl substituents, with the proviso that said phenothiazine must contain at least one additional five- to seven-membered ring, or a pharmaceutically acceptable salt thereof.

3. A method of inhibiting KSP kinesin comprising contacting KSP kinesin with a compound of formula



wherein

R^1 is hydrogen, halogen or CF_3;

R^2 is chosen from hydrogen and lower alkyl;

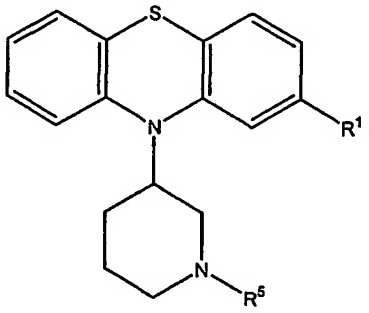
R^3 is hydrogen;

R^4 and R^5 are independently chosen from hydrogen, alkyl, substituted alkyl, alkylaryl, substituted alkylaryl, alkylheteroaryl and substituted alkylheteroaryl; or

any of R^2, $R^3$and R^4 taken together with the intervening atoms form one or more five- to seven-membered rings, said ring optionally substituted with one or more alkyl, aryl, alkoxy, halo, alkylaryl or substituted alkylaryl substituents, with the proviso that said phenothiazine must contain at least one additional five- to seven-membered ring, or a pharmaceutically acceptable salt thereof.

4. A method according to claim 1, 2 or 3 wherein wherein R^3 is hydrogen and R^2 and R^4 form a five- to seven-membered ring.

5. A method according to claim 4 wherein said phenothiazine has the formula



6. A method according to claim 1, 2 or 3 wherein R^2 and R^3 are hydrogen and R^5 is alkylaryl or substituted alkylaryl.

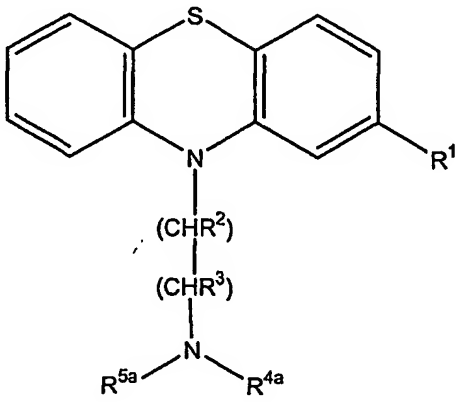
7. A method according to claim 1 or 2 wherein said disease or disorder is chosen from the group consisting of cancer, hyperplasia, restenosis, cardiac hypertrophy, immune disorders and inflammation.

8. A method according to claim 4 wherein said disease or disorder is chosen from the group consisting of cancer, hyperplasia, restenosis, cardiac hypertrophy, immune disorders and inflammation.

9. A method according to claim 5 wherein said disease or disorder is chosen from the group consisting of cancer, hyperplasia, restenosis, cardiac hypertrophy, immune disorders and inflammation.

10. A method according to claim 6 wherein said disease or disorder is chosen from the group consisting of cancer, hyperplasia, restenosis, cardiac hypertrophy, immune disorders and inflammation.

11. A phenothiazine of the formula



wherein

R^1 is hydrogen, halogen or CF_3;

R^2 is chosen from hydrogen and lower alkyl;

R^3 is hydrogen;

R^{4a} is chosen from hydrogen and lower alkyl; and

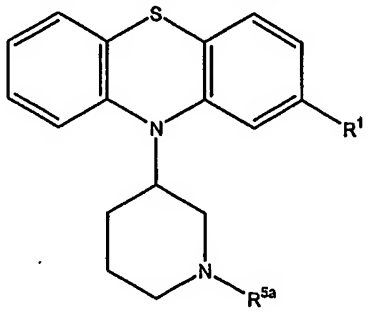
R^{5a} is chosen from alkylaryl, substituted alkylaryl, alkylheteroaryl and substituted alkylheteroaryl; or

any of R^2, R^3 and R^{4a} taken together with the intervening atoms form one or more five- to seven-membered rings, said ring optionally substituted with one or more alkyl, aryl,

alkoxy, halo, alkylaryl or substituted alkylaryl substituents, or a pharmaceutically acceptable salt thereof.

12. A phenothiazine according to claim 11 wherein R^3 is hydrogen and R^2 and R^{4a} form a five- to seven-membered ring.

13. A phenothiazine according to claim 12 of formula

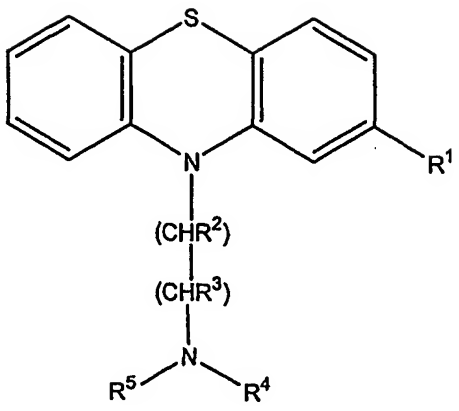


14. A phenothiazine according to claim 13 wherein R^{5a} is benzyl or substituted benzyl.

15. A phenothiazine according to claim 11 wherein R^2 and R^3 are hydrogen and R^{5a} is alkylaryl or substituted alkylaryl.

16. A phenothiazine according to claim 15 wherein R^{5a} is benzyl or substituted benzyl.

17. A method of screening for KSP kinesin modulators comprising:
combining a kinesin, a candidate bioactive agent and a compound of the formula



wherein

R^1 is hydrogen, halogen or CF_3;

R^2 is chosen from hydrogen and lower alkyl;

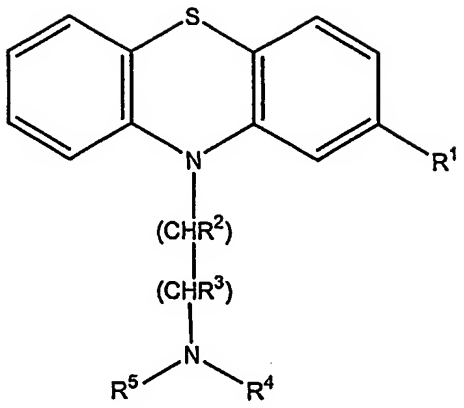
R^3 is hydrogen;

R^4 and R^5 are independently chosen from hydrogen, alkyl, substituted alkyl, alkylaryl, substituted alkylaryl, alkylheteroaryl and substituted alkylheteroaryl; or

any of R^2, R^3 and R^4 taken together with the intervening atoms form one or more five- to seven-membered rings, said ring optionally substituted with one or more alkyl, aryl, alkoxy, halo, alkylaryl or substituted alkylaryl substituents, with the proviso that said phenothiazine must contain at least one additional five- to seven-membered ring , , or a pharmaceutically acceptable salt thereof , and

determining the effect of said candidate bioactive agent on the activity of said kinesin.

18. A method of screening for compounds that bind to KSP kinesin comprising: combining a kinesin, a candidate bioactive agent and a labeled compound of the formula



wherein

R^1 is hydrogen, halogen or CF_3;

R^2 is chosen from hydrogen and lower alkyl;

R^3 is hydrogen;

R^4 and R^5 are independently chosen from hydrogen, alkyl, substituted alkyl, alkylaryl, substituted alkylaryl, alkylheteroaryl and substituted alkylheteroaryl; or

any of R^2, R^3 and R^4 taken together with the intervening atoms form one or more five- to seven-membered rings, said ring optionally substituted with one or more alkyl, aryl, alkoxy, halo, alkylaryl or substituted alkylaryl substituents, with the proviso that said phenothiazine must contain at least one additional five- to seven-membered ring, or a pharmaceutically acceptable salt thereof; and

determining the binding of said candidate bioactive agent to said kinesin.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 02/01710

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07D279/28 C07D279/26 C07D417/04 C07D417/14 A61K31/5415
A61P35/00 A61P37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BEILSTEIN Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2 784 185 A (ALFONS SCHULER WILHELM) 5 March 1957 (1957-03-05) the whole document, especially column 1, lines 1-45	1,11-16
X	US 2 830 987 A (GAILLOT P. ET AL) 15 April 1958 (1958-04-15) column 1; example 2	1,11,14
A	US 2 901 478 A (ALFONS SCHULER WILHELM) 25 August 1959 (1959-08-25) the whole document	1,11
A	EP 0 346 239 A (RHONE POULENC SANTE) 13 December 1989 (1989-12-13) claims	1,11

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 April 2002	10/05/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chouly, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 02/01710

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	WO 01 98278 A (BERGNES GUSTAV ;CHABALA JOHN C (US); FENG BAINIAN (US); FINER JEFF) 27 December 2001 (2001-12-27) claims	1,11
P,A	WO 01 30768 A (BERGNES GUSTAVE ;CHABALA JOHN C (US); FENG BAINIAN (US); FINER JEF) 3 May 2001 (2001-05-03) claims	1,11

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 02/01710

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(54) Title: FUNGICIDAL AND BACTERICIDAL COMPOSITIONS FOR PLANTS CONTAINING PHOSPHONATE AND PHOSPHATE SALTS, METAL CHELATES, AND DERIVATIVES THEREOF

(57) Abstract: The present invention relates to compositions containing at least one metal chelate, at least one phosphonate salt, and at least one phosphate salt, which are used as fungicides and bactericides, and methods of using the compositions.

FUNGICIDAL AND BACTERICIDAL COMPOSITIONS FOR PLANTS CONTAINING PHOSPHONATE AND PHOSPHATE SALTS, METAL CHELATES, AND DERIVATIVES THEREOF

RELATED APPLICATIONS

The present patent application is a continuation-in-part of U.S. Patent Application Serial No. 09/702,417, filed October 31, 2000, which is a continuation-in-part of U.S. Patent Application Serial No. 09/387,100, filed August 31, 1999 now U.S. Patent No. 6,139,879, which is a continuation-in-part of U.S. Patent Application Serial No. 08/881,968, filed June 25, 1997 now abandoned and which is a continuation-in-part of U.S. Patent Application Serial No. 09/419,127, which is a continuation-in-part of U.S. Patent No. 5,997,910, which is a divisional of U.S. Patent No. 5,800,837, which is a continuation-in-part of U.S. Patent No. 5,736,164.

FIELD OF THE INVENTION

The present invention is broadly concerned with fungicidal and bactericidal compositions, and methods of use, which provide improved efficacy in controlling fungus and bacterial infections in plants. More particularly, the compositions and methods relate to metal chelates, and preferably to a copper chelate in the form of Cu-EDDHA (copper ethylenediamine-di-o-hydroxyphenylacetic acid), including an effective amount of phosphate (PO_4) and phosphonate (PO_3), in aqueous solution.

BACKGROUND OF THE INVENTION

Fungicides, as well as bactericides, are either chemical or biological agents used to protect agricultural crops from infectious pathogens which, if left uncontrolled, result in the weakening or destruction of a plant. In regards to agricultural crops, this is unacceptable, as economic losses will result. Specific pathogens which tend to have an undesired effect on various agricultural crops include Citrus Greasy Spot, Citrus Melanose, Oak Leaf Blister, Erwinia, Xanthomonas, and Alternaria. In the interest of protecting valuable agricultural crops, it is desired to have a fungicide and bactericide composition that readily eliminates or treats these various plant maladies, as well as other infectious agents.

Copper (Cu) compounds that are active as fungicides and bactericides have been in agricultural use since the advent of Bordeaux in the grape vineyards of France in the early 1800s. It has been observed that various types of copper compounds can be used to effectively treat various plant pathogens. As such, many different formulations of fungicides employing copper compounds, such as wettable powders, water based flowables, and dry flowables, are commonly used today in modern agricultural applications. While copper compounds are known to impart desirable fungicidal and bactericidal properties, there are associated problems. Specifically, known copper compounds are typically either phytotoxic, non-soluble, or ineffective as a fungicide or bactericide.

Generally, copper compounds used as fungicides have, for the most part, been inorganic in form when applied to agricultural uses. The inorganic copper compounds have been used because they have been observed to be non-phytotoxic. Organic forms of the copper compounds, while beneficially water soluble, have been found to be generally phytotoxic, especially in foliar applications.

Water soluble, copper compounds such as $CuSO_4$, though effective to inhibit germination of fungus spores, when used in foliar applications to agricultural crops can be phytotoxic. Therefore, relatively insoluble forms of inorganic copper compounds, such as cupric hydroxide, have been found to be more effective fungicides. Note, however, that not all water insoluble Cu compounds are fungicidal or bactericidal. It is known that the in-vitro fungicidal activity is largely dependent on the copper solubility in the spore exudate and in the fungal cell. Also, despite the phytotoxicity, certain organic copper compounds have some utility as fungicides. An example of a suitable organic copper compound is CUTRINE (Cu salt of tri-ethanol amine) which is quite effective as an aquatic algicide, but unsuitable for use in other foliar applications.

While inorganic copper compounds are beneficially non-phytotoxic, they generally suffer from low water solubility. Modern day agricultural uses of inorganic copper compounds as fungicides employ varying forms of copper compounds having relatively low water solubility and include, for example, cupric hydroxide, tri basic copper sulfate, and tank mix combinations (with heavy metal ethylene-bis-di-thiocarbamate fungicides to

enhance the bactericidal activity against certain important agricultural bacterial such as Xanthomonas, Pseudomonas, and Erwinia). The lack of solubility of the inorganic copper compounds is an undesirable problem. Because known and popular copper fungicides are largely water insoluble, they are normally applied in relatively large volume aqueous suspensions and, as such, are readily removed by rain after application. Frequent applications are, thus, necessary at short intervals -- an application process which is expensive and environmentally imprudent.

Inorganic copper compounds alone are also not particularly effective in treating certain forms of fungus known as Phytophthora. From 1845 to 1846, the Irish Potato Famine occurred, which was one of the most devastating crop failures in the history of the world. The potato famine was caused by the disease late blight which resulted in harvested potatoes quickly decaying, making them unsuitable for consumption. The disease is also known to cause defoliation in infected plants. Late blight is caused by a *Phytophthora infestans* infecting potato and tomato plants. As can be gathered, the Phytophthora fungus, if not controlled, can cause major economic damage to agricultural crops, with the resulting damage causing the loss of millions of dollars in crop revenues. Additionally, there is the possibility of significant reduction of the potato and tomato supply available to consumers.

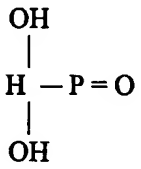
To control late blight, it has been recommended that the contaminated potatoes and/or tomatoes be buried in deep pits and covered by at least two feet of soil. In Northern Latitudes, the potatoes or tomatoes can be spread on the soil surface and allowed to freeze during the winter. These methods temporarily prevent the spread of the disease, but do not prevent infection and attack by the *Phytophthora infestans*. The treatment only addresses plants and crops after they have been destroyed. For this reason, it is desired to have a composition or method that can be administered to potato and tomato fields to actively control and prevent the spread of the *Phytophthora infestans* infestation.

Some species of the Phytophthora genus can be controlled, such as *Phytophthora parasitica*. In particular, fosetyl-al (ethyl phosphonate) can be administered to plants to control diseases such as root rot caused by *Phytophthora parasitica*. As such, it is known that many phosphonate (PO_3) compositions are highly effective in combating the disease root rot and, in particular, some of the species of the genus Phytophthora. Unfortunately, fosetyl-

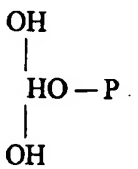
al and other phosphonates, alone, do not control late blight and similar Phytophthora diseases caused by the species *Phytophthora sojae*. Thus, it is desired to have a method or composition that readily inhibits infection by and proliferation of *Phytophthora infestans*.

Phosphorus is an essential element in plant nutrition because it governs the energy producing reactions, including those that are oxidative and photo phosphorylative. Phosphorous is essential to the production of adenosine diphosphate (ADP) and adenosine triphosphate (ATP). Energy-rich phosphate bonds of ADP and ATP provide the energy for many of the physiological reactions that occur in plants. As such, various forms of phosphorous are absorbed by plants for use as part of the photosynthetic process.

The element phosphorous appears in numerous general forms, including phosphonate (PO_3) and phosphate (PO_4). The term "phosphonate," sometimes also referred to as "phosphite," means the salts (organic or inorganic) of either phosphonic acid or phosphorous acid. Phosphonic and phosphorous acids have the formula H_3PO_3 and a molecular weight of 82.00. Their structures from the International Union of Pure and Applied Chemistry are shown below:

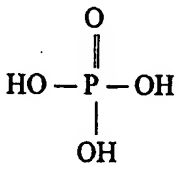


Phosphonic Acid
CA: 13598-36-2



Phosphorous Acid
CA: 10294-56-1

The term "phosphate" means the salts (organic or inorganic) of phosphoric acid having the formula H_3PO_4, molecular weight of 98.00 and having the following structure:



Phosphoric Acid
CA: 7664-38-2

In the past, various phosphonate compounds have been proposed as useful in fungicidal and fertilizer compositions for application to plants. See, e.g. U.S. Patent Nos. 4,075,324 and 4,119,724 to Thizy, describing phosphorous acid, its inorganic and organic salts, as a plant fungicide; U.S. Patent No. 4,139,616 to Dueret, describing fungicidal compositions based on phosphorous acid esters and salts thereof; U.S. Patent No. 4,542,023 to Lacroix et al., describing organophosphorous derivatives as possessing systemic and contact fungistatic and fungicidal activity; U.S. Patent Nos. 4,698,334, 4,806,445, and 5,169,646 to Horriere et al., describing fungicidal compositions based on alkyl phosphonates; U.S. Patent Nos. 4,935,410 and 5,070,083 to Barlet, describing fungicidal aluminum tris-alkyl-phosphonate compositions; and U.S. Patent No. 5,514,200 to Lovatt, describing formulations of phosphorous-containing acid fertilizer for plants. (The teachings of the proceeding U.S. Patents are hereby incorporated by reference.) The above references, disclosing phosphonate compositions, have been found to be effective for protecting plants and, particularly, grape vines, citrus and fruit trees, and tropical plants against fungal attack.

Note that phosphonate (PO_3) alone is typically considered an unacceptable source of phosphorus (P) for plants. It is known that PO_3 must be converted to PO_4 to be utilized by a plant.

Once assimilated, phosphonates (PO_3) have been shown to enhance the plant's phytoimmune system. The phosphonate induced stimulation of the phytoimmune system is triggered by the induction of ethylene production, followed by a rapid accumulation of phytoalexins at the site of infection. Phytoalexins are antibiotics which result from the interaction between the host plant and a pathogen. The phytoalexins are synthesized by and accumulate in the plant to inhibit the pathogen. The phytoalexins will accumulate at the site of an infection to prevent further spread of the disease, thereby reducing symptomatic expression of the disease.

In the past, phosphates (PO_4) were not viewed as a solution to pathological acerbation of fungal infections or infections produced by other genera. This is because phosphates (PO_4) are viewed primarily as a fertilizer with only limited, or even detrimental, phytoimmune properties. For example, U.S. Patent 5,514,200 teaches that phosphate fertilizers inhibit beneficial symbiosis between plant roots and mycorrhizal fungi, and further promote bacterial

and fungical growth in the rhizosphere, including the growth of pathogenic fungi and other soil-borne organisms. (Col. 2, lines 18-28). Phosphates (PO_4) have also been considered to be a competitive inhibitor for phosphonate assimilation, thus inhibiting the ability of phosphonates (PO_3) to protect against fungus attack. See, Pegg, K.G. and deBoer, R.F., "Proceedings of the Phosphonic (Phosphorous) Acid Work Shop," *Australiasian Plant Pathology*, Vol. 19 (4), pp. 117 and 144, 1990. Yet further, phosphonates (PO_3) and phosphates (PO_4) were believed to be "biological strangers," with the presence of phosphonates (PO_3) or esters of phosphonates, exerting little or no influence on enzyme reactions involving phosphates. Robertson, H.E. and Boyer, P.D., "The Biological Inactivity of Glucose 6 — phosphonate (PO_3), Inorganic Phosphites and Other Phosphites," *Archives of Biochemistry and Biophysics, 62 pp. 380 - 395 (1956).*

Thus, both forms, inorganic and organic Cu compounds, as well as phosphates (PO_4) and phosphonates (PO_3) when used individually, suffer from problems. Therefore, the need exists for a highly water soluble Cu compound based fungicide and bactericide that is not phytotoxic. A need also exists for a water soluble Cu compound based fungicide and bactericide that reduces the adverse Cu load on the plant, thus reducing the non-target impact to the environment. Further, a need exists for such fungicidal and bactericidal compounds that permit use of other metals such as manganese, zinc, iron, copper and mixtures thereof, as may be desired for specific fungicidal or bactericidal properties. Also, the requirements for a successful phosphonate-based fungicide depend on the promotion of the phosphonate-induced pathological acerbation of fungical or other genus infections. More particularly, it is desired to have a composition and/or method that prevents *Phytophthora infestans* infection and destruction of plants.

SUMMARY OF THE INVENTION

The present invention relates to a metal chelate fungicide and bactericide composition that also includes effective amounts of phosphate (PO_4) and phosphonate (PO_3), and methods of using the composition to control fungicidal and/or bactericidal infection in plants. Preferably, the chelate is a composition that is a member of the EDDHA (ethylenediamine-di-o-hydroxyphenylacetic acid) family, and the metal is selected

from the group consisting of iron, copper, manganese, zinc, tin, and combinations thereof. Copper, however, is the most preferred metal. Importantly, the present invention addresses the problems discussed before, of solubility, phytotoxicity, and effectiveness. In particular, the fungicide and bactericide composition of the invention provides an improved antifungal and antibacterial composition for use on plants that contains, as active ingredients, fungicidally and/or bactericidally effective amounts of the metal chelates, phosphonates, and phosphates in aqueous solution. It has been observed that the application of the composition of the invention to a plant substantially eliminates fungus and bacteria disease. Not only is the composition effective in eliminating fungus and bacteria, but it is substantially non-phytotoxic. Also, the metal chelate in the composition of the invention is soluble in aqueous solution. Thus, the composition provides for protection of plants against fungal and bacterial infections without the attendant phytotoxicity.

Importantly, the composition of the invention is a singular product that imparts antifungal and antibacterial protection upon application without being phytotoxic. The composition of the invention is, additionally, environmentally safe, comparatively inexpensive to use, and has low mammalian toxicity.

Essentially, the present antifungal and bacterial composition is comprised of an active material, a fungicidally and/or bactericidally effective amount of a metal chelate, and an agriculturally acceptable carrier, such as water. The preferred fungicidal and bactericidal compositions is comprised of water and a metal chelate selected from the group consisting of Fe-EDDHA (iron ethylenediamine-di-o-hydroxyphenylacetic acid), Cu-EDDHA, Mn-EDDHA, Zn-EDDHA, Sn-EDDHA, and mixtures thereof. Other family members of EDDHA can be substituted therefor, including pEDDHA and EDDHMA. Desirably, both antifungal and antibacterial effects are achieved with one composition.

The composition of the invention also includes phosphate (PO_4) and phosphonate (PO_3) constituents which, when combined, particularly provide for a synergistic effect that results in the substantial protection against infection of plants by Phytophthora, especially *Phytophthora infestans*. As such, the phosphate and phosphonate constituents can be combined to form a composition, which can be applied to plants, especially tomatoes and

potatoes, to prevent infection by *Phytophthora infestans* and diseases caused by such infection. Application can be achieved by using either a dry mix or an aqueous solution.

The preferred composition for preventing Phytophthora will be comprised of at least one potassium phosphonate and at least one potassium phosphate, as it has been found that these two constituents, when combined, will cause a synergistic effect which results in the substantial prevention of infection by Phytophthora. It is believed, that the rate by which infection is prevented is increased by at least 100% when the two constituents are combined, as compared to the additive effect of the combined salts. The two constituents will be combined in an amount sufficient to prevent infection and manifestation by various disease causing organisms, with the particular amounts combined dependent upon the particular species of plant to be treated, the specific disease causing organism to be treated, and the particular phosphate salt and phosphonate salt that will be combined.

The composition should be applied at least once to the plants to be treated. While one application is sufficient, it is typically preferred to make multiple applications. Essentially, any plant infected by Phytophthora can be treated, with it most preferred to apply the composition to potato and tomato plants. It should also be noted that the composition not only inhibits Phytophthora, but is environmentally safe, inexpensive to use, and has low mammalian toxicity.

Phosphonate salts useful in the practice of the invention also include those organic and inorganic salts taught by U.S. Patent Nos. 4,075,324 and 4,119,724 to Thizy et al., (see, e.g., col. 1, ln. 51-69 through col. 2, ln. 1-4).

DETAILED DESCRIPTION

The present invention relates to a composition that is both a fungicide and a bactericide and a method for using such composition. The composition is advantageously useful in eliminating or at least substantially reducing the effects of infection by various fungal and bacterial plant pathogens. The composition of the invention contains at least one metal chelate in aqueous solution, with it preferred that the chelate be a member of the EDDHA family of compositions, at least one phosphonate salt, and at least one phosphate salt. The metal attached to the chelate can be selected from any of a variety of metals,

especially those selected from rows 4 or 5 of the periodic table of the elements, particularly metals of row 4.

As stated, one component of the present composition is a metal chelate, preferably in an amount of water to form an aqueous composition. The metal chelate can be formed by a known process, with the reaction summarized as reacting an amount of metal chloride hexahydrate with water and an amount of mono-amide di-hydrochloride in a reaction vessel. A catalyst, such as sodium hydroxide, is then added which will cause formation of the metal oxide EDDHA. Among the metal hexahydrates that can be reacted with the mono-amide di-hydrochloride are iron, zinc, tin, manganese, and copper, preferably zinc, manganese, and copper, and more preferably copper. The resulting metal chelates that are suitable for use include: Fe-EDDHA, Cu-EDDHA, Zn-EDDHA, Mn-EDDHA, Sn-EDDHA, and combinations thereof. In addition, the corresponding metal chelates of para-ethylenediamine-di-o-hydroxyphenylacetic acid (pEDDHA), ethylenediamine-di-o-hydroxyphenylmethylacetic acid (EDDHMA), and combinations thereof are also particularly suitable for use.

The term "metal chelate" refers to an organic coordination "complexing" compound in which a metal ion is bound to atoms of non-metals, e.g., nitrogen, carbon, or oxygen, to form a heterocylic ring having coordinate covalent bonds. The non-metal atoms may be attached to the metal ions by from one to six linkages and, thus, are called uni, bi, tri dentate, etc., meaning 1-, 2-, or 3-tooth. Suitable metals commonly involved in chelate structures include those metals selected from rows 4 or 5 of the periodic table of the elements, particularly metals of row 4. Examples of suitable metals include, but are not limited to, cobalt, copper, iron, nickel, zinc, tin, and manganese, preferably copper, iron, zinc, and manganese, more preferably copper, zinc, and manganese, and most preferably copper. Examples of specific metal chelate structures include:

Fe-HEEDTA (hydroxyethylethylenediaminetriacetic acid), Fe-EDTA (ethylenediaminetetraacetic acid), Fe-DTPA (di-ethylenetriaminepentaacetic acid), Fe-EDDHA (ethylenediamine-di-o-hydroxyphenylacetic acid), ethylene-bis-di-thiocarbamates of Mn and Zn (EBDC), Cu-EDDHA, Mn-EDDHA, and Zn-EDDHA.

To form the metal chelate aqueous composition the metal chelate will be mixed with an amount of water to form an aqueous solution. Generally, special treatment of the water is not required, such as deionizing the water for example. Additionally, the mixing will preferably occur under ambient conditions. The metal chelate will be mixed into the water in an amount sufficient to cause the finished composition to equal from about 0.01 pounds to about 2.0 pounds of AI (active ingredient, i.e. the metal) per acre. Preferably, the amount of metal chelate is about 0.01 to about 0.8, and more preferably about 0.01 to about 0.2 pounds of AI per acre. Typically this means adding the metal chelate to the water in an amount equal to between about 1% and about 5% by weight (on a metal basis) of the total solution. More preferably, the metal chelate will be added in an amount equal to between about 2% and about 4% by weight, and most preferably about 3% by weight of the total solution.

Once the aqueous composition has been formed by thoroughly blending the phosphonate salt, phosphate salt, and metal chelate with the water, the aqueous composition is then ready for application to plants, in particular agricultural crops. The aqueous composition is typically easily applied by spraying or other means of distributing the aqueous solution in a sufficient amount to the plants.

The metal chelate must be applied in a sufficient AI amount, without resulting in phytotoxicity. Unacceptably high levels of phytotoxicity result in foliar burn, defoliation and stem die-back, necrosis, plant stunting, or death. Phytotoxicity can be rated on an international scale of 0-10 where 0 is equal to no phytotoxicity and 10 is complete death of the plant. It is preferred if the metal chelate is applied in an amount so that the phytotoxicity is minimized.

Phytotoxicity rankings of Fe chelates, for example, used in foliar applications are as follows: Fe-HEEDTA -- most phytotoxic; Fe-EDTA -- intermediate phytotoxic, Fe-DTPA -- less phytotoxic, and Fe-EDDHA -- least phytotoxic. Thus, the Fe-EDDHA is preferred because it is the least phytotoxic while still imparting excellent fungicidal and bactericidal properties.

Metal chelates disclosed herein will have a water solubility acceptable for use in the inventive fungicide and bactericide. For example, the solubility of sequestrene 138 Fe Iron

Chelate in pounds per 100 gallons of water, at various temperatures is similar to the present metal chelate in aqueous solution. (Solubility weight/100 gals. H_2O) is shown in Table 1 below:

Temperature (°C)	Lbs.	Oz.
0	69	11
10	70	7
20	75	4
30	81	11
40	84	1
50	88	1

Commercially produced Sequestrene 138 Fe contains 6% Iron as metallic, or 8.5% iron as Fe_2O_3. The commercial product has a moisture content of not more than 10%. As such, this is exemplary of a suitable solubility. Thus, it is desired for the metal chelate, in particular the Cu-EDDHA, to have a solubility of about 100% where at least 80 lbs. of metal chelate is dissolved in 100 gallons of H_2O at 50° C.

Without being limited to this theory, it is believed that metal chelation generally increases the water solubility of the metal ion and the availability in certain soil conditions of the metal ion where calcareous and high pH situations would otherwise prevent metal ions from being available to the plant as a fungicide.

It is believed that certain metal chelates (usually in the form of Mn, Zn, and Fe) may be applied foliarly at much reduced rates when compared to inorganic salts intended for fungicidal use.

The preferable method of application is foliar, either by ground or aerial equipment, but is not limited to that method alone. Injection or soil applications, for example, can also be used depending upon the specific crops and pathogens.

Among the plants that can be treated with the metal chelate in aqueous solution are: fruit crops, and agronomic crops, ornamentals, trees, grasses, vegetables, grains, and flori-cultural crops, as well as, some aquatic crops, including rice.

The fungicidal and bactericidal properties of the compounds according to the invention are various, but are particularly interesting in the cases described in the following examples.

The following examples set forth preferred concentrations and techniques for formulation thereof, as well as methods of application and use in test results, demonstrating the efficacy of the inventive concentration in protecting plants against attack by fungi or bacteria, or both. It is to be understood, however, that these Examples are presented by way of illustration only, and nothing therein shall be taken as a limitation upon the overall scope of the invention. The specific components tested in the Examples were prepared and applied as follows:

To prepare Cu-EDDHA, an appropriate Cu salt need merely be substituted for the iron salts as discussed before and disclosed in U.S. Patent No. 2,921,847, which is incorporated herein by reference.

As used in the Examples, "Ave. % infection" means percent of leaves that exhibit fungus lesions.

EXAMPLES

Example 1.

Cu-EDDHA and four commercially accepted fungicidal compositions were applied to Valencia orange on sour orange rootstock. Applications were in 100 gallons of solution (in the concentrations indicated in the table below) per acre in mid-summer to single-tree plots replicated six times in a randomized complete block ("RCB") design. Seven months later, the percentages of Citrus Greasy Spot infection on five branch terminals from each tree were recorded and averaged.

CITRUS GREASY SPOT TEST
CAUKINS GROVES, INDIANTOWN, FLORIDA

TREATMENT	RATE/100 GALLONS	*AVE% INFECTION 2/10/88
FCC-455 Spray Oil (Florida Citrus Commission)	1%	30.0
Difenconazole	50 gram (gm) AI	1.56
Difenconazole	100 gm AI	1.0
Cu-EDDHA 3.2%	0.2 lbs. AI	2.5
KOCIDE 101	4 lbs.	23
Untreated	--	35

APPL. Single tree plots x 6 Reps.
* Aug. 5 terminals/tree

The Difenconazole (triazole): specifically, 1-2[2-[4-(4-chlorophenoxy)-2-chlorophenyl-(4-methyl-1, 3-dioxolan-2-l)-methyl]]-1H-1,2,4-triazole (available from Ciba-Geigy, Greenborough, North Carolina) is a triazole fungicide. As can be seen, from the table it provided desirable fungus repression. But, triazole is well known to be potentially hazardous to human health, in particular it is known to be damaging to the human liver.

Also tested, was Cu-EDDHA: (Sodium cupric ethylenediamine-di-o-hydroxyphenylacetic acid), which is the fungicide/bactericide of the present invention. Analysis of the data shows excellent results from applying the Cu-EDDHA.

KOCIDE 101 is a fungicide available from Griffin Corp., Valdosta, Georgia. The composition FCC-455 spray is also fungicide. The % infection of the plants treated with the KOCIDE 101 and FCC-455 compositions is considered unacceptable. Also, note that KOCIDE 101 is a copper hydroxide composition.

From the table AVE % infection relates to the percentage infection of Citrus Greasy Spot (*Mycosphaerella citri*) found on the treated leaves.

Example 2.

In the present Example, fungicides were again tested on "Valencia" oranges except the effect of various fungicides on perithecia was tested. Three different fungicides, the Cu-EDDHA, TILT (Propiconazole made by Ciba-Geigy), and difenconozole were applied in 100 gallons per acre (gpa) to single tree plots of "Valencia" oranges replicated five times in a RCB design in mid-July.

Twenty mature leaves (from the spring flush) per replicate were harvested approximately 4 months later and placed under greenhouse conditions and alternately wetted and dried to simulate natural defoliation and weathering.

These conditions, in turn, cause the fungus to sporulate by the formation of perithecia (spore production body of fungus). The spores were counted as a means of measuring the fungicidal activity of the treatments. The data is presented below.

CITRUS GREASY SPOT

SCN NURSERY, DUNDEE, FLORIDA

TREATMENT	RATE/100 GALLONS	# PERITHECIA
Cu-EDDHA 3.2%	0.2 lbs. AI	3.24 b
Cu-EDDHA 3.2%	0.4 lbs. AI	5.93 ab
TILT 3.6 EC	6 oz. Prod.	6.62 ab
Difenconazole	100 gm AI	5.32 ab
Difenconazole	200 gm AI	11.57 ab
CONTROL inoculated		7.97 ab
CONTROL not inoculated		6.42 ab

The conditions of the test were as follows:
Function: ANOVA - 1
Date Case No. 1 to 42
Without selection
One way ANOVA grouped over variable 1
TREATMENT NUMBER
With values from 1 to 7
Variable 3
NUMBER OF PERITHECIA PER 5 MM FIELD AT 2.5 X -- MEAN OF THREE OBSERVATIONS

As used herein, a, b, c, and ab indicate statistical significance using Duncan's multiple range test. In interpreting the data, a different notation, e.g. b versus ab, means there was a statistically significant difference in the results. A difference in data of samples with the same letter notation was not statistically significant.

The tests produced important data because if the perithecia is reduced then it follows that the number of infections are reduced. The Cu-EDDHA showed good results. The TILT also showed decent results, but is not preferred because it has limited uses as promulgated by the FDA (Food and Drug Administration). Also, note that Cu-EDDHA added in a higher AI did not result in enhanced repression of the perithecia. This seems to indicate that if too much Cu-EDDHA is added, slight phytotoxicity will result.

ANALYSIS OF VARIANCE TABLE

	DEGREES OF FREEDOM	SUM OF SQUARES	ERROR MEAN SQUARE	F-VALUE	PROB.
Between	6	226.6508	37.78	1.33	.270
Within	34	965.0170	28.38		
Total	40	1191.6678			

Example 3.

Cu-EDDHA, KOCIDE 101 (cupric hydroxide), and difenconazole were applied to single tree plots of "Hamlin" oranges in 100 gpa (in concentration indicated) in a RCB design replicated 4 times. Applications were made in either May, June or May, and June. Ten fruit/replicates were sampled in July and percent infection of Melanose (Diaporthe citri) was determined. See data presented below.

CITRUS MELANOSE CONTROL
R.E. KEENE FRUIT COMPANY

TREATMENT	RATE, LB AI/100 GALLONS	TIMING	% INFECTION (FRUIT)
Cu-EDDHA 3.2%	0.2	May	9
Cu-EDDHA 3.2%	0.4	May	13
Cu-EDDHA 3.2%	0.8	May	21
Cu-EDDHA 3.2%	0.2	May - June	11
Cu-EDDHA 3.2%	0.4	May - June	15
Cu-EDDHA 3.2%	0.8	May - June	29
Cu-EDDHA 3.2%	0.2	June	14
KOCIDE 101	4.0	May	12
KOCIDE 101	0.4	May - June	10
DIFENCONAZOLE	0.5	June	4
Untreated	--	--	38

4 REPS SINGLE TREE PLOTS.

PENETRATOR (surfactant - non-ionic) @ 4 oz. ALL TREATMENTS

The results indicate that in general not too much Cu-EDDHA should be applied to the plants. Also, an appropriate application time of year such as May, should be chosen.

Example 4.

In the present example, GRAPEFRUIT plants (*Citrus paradisi* 'Marsh') were tested with various fungicides to determine the effectiveness in eliminating greasy spot, *Mycosphaerella citri*.

Spray treatments were applied dilute (applied to point of run off) by handgun in July to 10-foot high trees at a rate equivalent to 700 gpa. Treatments were replicated on 8 single tree plots in a RCB design. Groups of 15 shoots on each of the east/west and east side of each tree were tagged and the initial number of leaves was recorded. In February, remaining leaves were counted and examined for greasy spot.

TREATMENT AND RATE/100 GALLONS	DEFOLIATION	% REMAINING LEAVES WITH GREASY SPOT
Tribasic copper sulfate (53% Cu) 0.75 lb.	1.9 a	20.1 a
Sunspray 7E oil 1 gal.	3.1 a	27.0 a
Difolatan 80 Sprills 1.25 lb.	8.9 b	49.8 b
Spotless 25W 0.8 lb	1.3 a	22.6 a
Tilt 3.6EC 8 fl. oz.	1.5 a	15.9 a
Cu-EDDHA (3.2% Cu) 1.5 gal.	0.8 a	12.0 a
Untreated	9.7 b	48.5 b

All treatments, except Difolatan (fungicide), reduced greasy spot-induced defoliation and the percentage number of remaining leaves with greasy spot symptoms. There were no significant differences in effectiveness between Tribasic copper sulfate, spray oil, Spotless, Tilt, and Cu-EDDHA. There was too little greasy spot rind blotch in this test to provide information on the relative efficacy of treatments for preventing fruit infection.

Copper sulfate is phytotoxic so that it needs to have its phytotoxicity reduced. This is accomplished by combining CaOH with the $CuSO_4$. Unfortunately, this reduces solubility.

Example 5.

Cu-EDDHA, TILT (propinconazole), difenconazole, and MERTECT ((Merck Chem., N.J.) thiabendazole) were applied in 100 gpa to 2-year-old laurel oaks (Quercus hemispherica) in 2 x 2 gal. pots in a RCB design replicated 4 times. MERTECT is a standard well known fungicide that does not include copper. Applications were made in July approximately 3 weeks apart and rated in August a month later. See data below.

OAK LEAF BLISTER (Taphrina Caerulescens) CONTROL
TRAILRIDGE NURSERY, KEYSTONE HEIGHTS, FLORIDA

TREATMENT	RATE, PRODUCT/ 100 GALLONS	*DISEASE INDEX
Tilt 3.6 emulsifiable	8 oz.	1.5
Difenconazole 3.6 emulsifiable	2 oz.	2.25
Cu-EDDHA 3.2%	8 oz.	2.8
MERTECT	8 oz.	1.5
Untreated	--	4.25

*Disease Index:
1 = no disease
2 = light
3 = moderate
4 = heavy
5 = dead foliage

2 x 2 gal. trees/exp. unit x 4 Reps in a RCB design

As can be seen, suitable disease repression occurred with the Cu-EDDHA composition, even though the concentration is higher than the preferred amount.

Example 6.

Cu-EDDHA and Kocide (cupric hydroxide) were applied as foliar spray in May to *Hibiscus sinensis* cuttings (100/replicate) x 4 replicates in a RCB design. Treatments were allowed to dry for one hour and then placed in a commercial propagation bed under intermittent mist and rated for bacterial (*Erwinia chrysanthemi*) infection one week later. Data is presented below:

***ERWINIA CONTROL ON HIBISCUS**

NELSONS NURSERY, APOPKA, FLORIDA

TREATMENT	RATE, CU/ 100 /GALLONS	AVERAGE % INFECTION
Cu-EDDHA 3.2%	0.2 lb. AI	6
Cu-EDDHA 3.2%	0.4 lb. AI	8
Kocide 101	2 lbs. AI	25
Untreated	--	100

100 Cuttings/REP X 4 *ERWINIA Chrysanthemi

The Cu-EDDHA added in an amount equal to 0.2 lb. AI showed excellent control of Erwina on Hibiscus.

Example 7.

A follow-up experiment to Example 6 was conducted on rooted cuttings which were dipped as they were removed from the propagation bed and foliarly sprayed 7 days later after being potted. Cu-EDDHA and Kocide 101 were applied at the rates specified below in a RCB design utilizing 100 plants/replicate x 4 reps. Potted cuttings had not received any previous bactericide treatments prior to potting.

ERWINIA CONTROL ON HIBISCUS
NELSONS NURSERY -- APOPKA, FLORIDA

TREATMENT	RATE, LB. AI/ 100 GALLONS	AVERAGE % INFECTION
Cu-EDDHA 3.2%	0.2	19
Cu-EDDHA 3.2%	0.8	32
KOCIDE 101	2.0	22

APPLIC. DATES: 7/19 DIP, 7/26/85 SPRAY
100 PLANTS/REP. X 4

Again excellent control was achieved with Cu-EDDHA applied in an amount equal to 0.2 lb. AI.

Example 8.

The present Example relates to controlling bacterial spots on pepper plants. The procedure for the present Example was as follows: Early Cal Wonder variety pepper plants were treated at weekly intervals with the following bactericides (g AI/liter in parentheses): copper + mancozeb (2 + 1), Cu-EDDHA (0.1), CGA (Ciba Geigy American)-115944, CGA-151731, CGA-157566, and CGA-164058 (each at 0.25 and 0.5), CGA-143268 (1.0). Treatments were applied weekly in 1000 1/ha for a total of eight applications. The crop was artificially inoculated after the first and third applications. Disease severity was evaluated after the fourth and eighth applications. Phytotoxicity was rated after the eighth application and yields were taken continually during the test.

The results of the testing were determined as such: Disease pressure was moderate and uniform. After four applications, the best treatments were CGA-115944, CGA-151731, and CGA-164058. CGA-157566 was less effective than the three previously mentioned compounds, but more effective than CGA-143268 which was equal to copper plus mancozeb and Cu-EDDHA in activity. The ranking of compounds changed when treatments were rated 12 days after the last application. Copper plus mancozeb control has completely broken down, which was expected because disease conditions were severe in the final half of the test and copper should be applied on a five-day schedule under these conditions. Cu-EDDHA at only 0.05X the rate of Kocide 101 (on a metallic copper basis) exhibited some control and was equal to CGA-143268, CGA-157566, and CGA-164058. The best bactericide at the second rating were CGA-115944 and CGA-151731. The phytotoxicity of all treatments was assessed after eight applications had been made. The only bactericides which were phytotoxic were CGA-115944 and CGA-164058. CGA-164058 was safer than CGA-115944, which was marginally unacceptable at 0.5 g AI/1. CGA-143268 and CGA-164058 increased yields dramatically. Yields were depressed by CGA-115944, CGA-151731, and CGA-157566. Cu-EDDHA had no effect on yield and copper + mancozeb increased yields moderately.

Example 9.

Croton plants inoculated with Xanthomonas were tested with various fungicides. Cu-EDDHA at 0.2 and 0.4 lbs. AI/100 gal. and Kocide 101 at 7.4 lbs. AI/Acre (A) were applied as foliar applications to croton (*Codiaeum variegatum*) previously inoculated with *Xanthomonas campestris* a day earlier. Treatments were assigned in a RCB design and replicated 10 times with single pots. Treatments were applied 3 times on a weekly schedule and evaluated at 7 and 14 days following the last application. See data below.

Test 1 Codiaeum Inoculated with Xanthomonas
Number of leaves with symptoms

TREATMENT	RATE, AI/100 GALLONS	AVERAGE % OF INFECTION
Water	noninoculated	0 a
Water	inoculated	2.6 c
Cu-EDDHA 3.2%	26 ml (.2 lb.)	.6 ab
Cu-EDDHA 3.2%	52 ml (.4 lb.)	1.0 b
Kocide 101	6.8 ml. (7.4 lb.)	.9 ab

ANOVA table

SOURCE	SUM OF SQUARES	DF	MEAN SQUARE	F VALUE
Treatment	37.28	4	9.319	9.177
			significant at 1% level	
Error	45.7	45	1.016	
Total	82.98	49		

It was determined that all of the copper treatments provided some control of Xanthomonas leaf spot of Codiaeum, when compared to the inoculated control. The lower rate of Cu-EDDHA and the Kocide 101 gave control equal to the noninoculated control treatment.

Example 10.

In the present example, carrots were inoculated with the Alternaria fungus. The carrots were then treated with the below listed fungicidal compositions. Also a control test was conducted. The percentage of the fungicide in the solution is also listed below along with the results. Plot size for testing was a single row of 25 feet by 4 repetitions in an RCB design. The fungicide was applied eight (8) times with the carrots then examined for infection seven (7) days and twenty-five (25) days after the last fungicidal treatment.

CARROT/ALTERNARIA FUNGICIDE TRIAL

	Treatment	Rate, AI/100 Gallons	Average % of Infection 04/05/96	04/23/96
1)	K_2HPO_4	1 gal. (0.53 wt. % AI)	6.9	8.2
2)	K_2HPO_3	1 gal. (0.55 wt. % AI)	18.7	28.8
3)	K_2HPO_4 + K_2HPO_3	0.5 gal.(0.26 wt. % AI) + 0.5 gal. (0.27 wt. % AI)	8.9	10.7
4)	Cu-EDDHA	0.2 lb AI	8.8	11.6
5)	Fe-EDDHA	0.2 lb AI	12.7	12.9
6)	CONTROL	—	23.0	34.8

*EDDHA (Ethylenediamine-di-o-hydroxyphenylacetic acid)

PLOT SIZE: Single Row X 25 ft. X 4 reps in a RCB design.

Application dates: 2/2, 9,15,22,3/8,14,22, and 28. Rated 4/5 and 4/23/96

NOTE: Second rating was 25 days after last fungicide application. Plots were inoculated with *Alternaria dauci*.

The tests were conducted in Sanford, FL.

As can be seen, Cu-EDDHA effectively limited percentage of fungicidal infection. It can be concluded that the Cu-EDDHA as well as the Fe-EDDHA are effective fungicides.

The above Examples demonstrate that the EDDHA metal chelate compositions are useful in protecting plants against attack by fungus with the application of the EDDHA metal chelate solution.

It will be further appreciated that foliar application of the EDDHA metal chelate compositions will be effective as a common agricultural practice to control bacterial infections in plants.

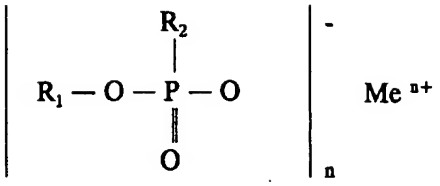
It will be appreciated by those skilled in the art that beneficial effects demonstrated in the Examples by the use of Cu-EDDHA will also be obtained when the Mn, Sn, Fe, and Zn EDDHA form metal chelates are employed.

The present invention also relates to compositions and methods for use in preventing diseases, such as late blight, caused by the genus Phytophthora. In particular, the present invention relates to compositions and methods for use in

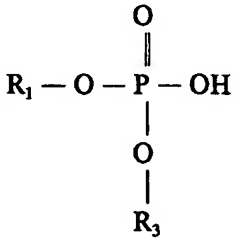
preventing plant diseases caused by *Phytophthora infestans*. The composition of the invention comprises at least one phosphate (PO_4) constituent and at least one phosphonate (PO_3) constituent in addition to at least one metal chelate, with it most preferred that a composition comprising at least one potassium phosphonate and at least one potassium phosphate be used. A mixture of mono- and dipotassium phosphonate salts can be readily used as the potassium salt, and a mixture of mono- di- and tripotassium phosphate salts can be readily used as the phosphate salt. Once the composition is formed, it can be applied to plants to prevent infection by *Phytophthora infestans* and manifestations related to the infection. The composition can be applied as either a dry mix or an aqueous solution to plants prior to infection by the *Phytophthora infestans* organism.

The composition for preventing *Phytophthora infestans* contains a combination of phosphonate and phosphate constituents. Any of a variety of phosphates are suitable for use, including K_2HPO_4, K_3PO_4, KH_2PO_4, $(NH_3)_2\ HPO_4$, $(NH_3)\ H_2PO_4$, and combinations thereof. The phosphonates, like the phosphates, can be selected from any of a variety of compositions, including K_2HPO_3, $KH_2PO_3\ (NH_3)_2\ HPO_3$, $(NH_3)\ H_2PO_3$, and combinations thereof. Any phosphate and phosphonate constituent combination can be used as long as infection by and manifestation of Phytophthora infestations is inhibited. Additionally, it is necessary for the constituents to have suitable solubility in a carrier and to be of a constitution to allow easy distribution in an area where plants to be treated are grown. More preferably, the phosphonate and phosphate constituents, when combined, will have a synergistic effect in inhibiting *Phytophthora infestans*. The most preferred phosphate (PO_4) and phosphonate (PO_3) constituents for use in preventing *Phytophthora infestans* infection are combinations of K_2HPO_3 and K_2HPO_4. As such, the phosphate (PO_4) and phosphonate (PO_3) constituents are combined to form the composition used to prevent *Phytophthora infestans* infection.

While the discussed constituents are preferred for use in treating plants and preventing infection by the Phytophthora organism, variations of the phosphate and phosphonate constituents can be used. As such, it is preferred if the compound comprises a fungicidally effective amount of at least a first salt having the following formula:



and a second salt having the following formula:



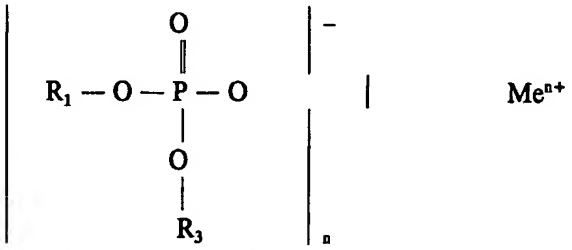
where R_1 is selected from the group consisting of H, K, an alkyl radical containing from 1 to 4 carbon atoms, halogen-substituted alkyl or nitro-substituted alkyl radical, an alkenyl, halogen-substituted alkenyl, alkynl, halogen-substituted alkynl, alkoxy-substituted alkyl radical, ammonium substituted by alkyl and hydroxy alkyl radicals;

R_2 and R_3 are selected from a group consisting of H and K;

Me is selected from a group consisting of K, alkaline earth metal cations, aluminum atom, and the ammonium cation; and

n is a whole number from 1 to 3, equal to the valence of Me.

Optionally, the second salt can be of the formula:



with the above listed formula constituents still applicable.

The constituents should be preferably mixed with a suitable carrier to facilitate distribution to an area where the plants to be treated are grown. The carrier should be agriculturally acceptable, with water (H_2O) most preferred.

As an example of how to form the composition, it is preferred to first form a potassium phosphonate aqueous solution, with the phosphonate formation as follows:

H_3PO_3 is produced by the hydrolysis of phosphorus trichloride according to the reaction: $PC1_3 + 3H_2O \rightarrow H_3PO_3 + 3HCl$. The HCl is removed by stripping under reduced pressure, and the phosphonic acid (H_3PO_3) is sold as a 70% acid solution.

The phosphonic acid is then neutralized in aqueous solution by potassium hydroxide according to the reactions: $H_3PO_3 + KOH \rightleftharpoons KH_2PO_3 + H_2O$

$KH_2PO_3 + KOH \rightleftharpoons K_2HPO_3 + H_2O$

to about pH 6.5, and to produce a 0-22-20 liquid weighing 11.15 lbs./ gal. This solution is commercially available and is sold under the trademark "Phos-Might" by Foliar Nutrients, Inc., Cairo, GA 31728.

The phosphate (PO_4) is produced by reacting mono potassium phosphate (0-51.5-34) with 45% potassium hydroxide in aqueous solution to produce dipotassium phosphate, by the following reaction: $KH_2PO_4 + KOH \rightleftharpoons K_2HPO_4 + H_2O$ with a product density of 1.394 g/mL at 20° C and a solution pH of 7.6 producing a 0-18-20 analysis. This solution is commercially available and is sold under trademark "K-Phos" by Foliar Nutrients, Inc., Cairo, GA 31724.

After the potassium phosphonate and potassium phosphate constituents, or other phosphonate and phosphate constituents, are formed, they can be combined to produce the potassium phosphonate and potassium phosphate composition, e.g. a mixture of the potassium salts of PO_3 and PO_4. The phosphonate and phosphate composition can then be combined with the metal chelate to form the composition of the invention. This composition is used to then treat plants for the prevention of infection by the Phytophthora genus, especially *Phytophthora infestans*.

Varying amounts of each compound, for example, K_2HPO_3, KH_2PO_3, K_2HPO_4, or KH_2PO_4 in an aqueous solution, are combined at rates ranging from 0.1 millimolar to 1000 millimolar, preferably 1 millimolar to 500 millimolar, more preferably 5 millimolar to 300 millimolar, and most preferably 20 millimolar to 200 millimolar, depending on crop host and the pathogen complex and level of infection. A 5% vol./vol. aqueous solution of K_2HPO_4 is equivalent to 2.6% by weight and is approximately 151 millimolar, and a 5% vol./vol. aqueous solution of K_2HPO_3 is equivalent to 2.7% by weight and is approximately 173 millimolar. A 20 millimolar aqueous solution of K_2HPO_4 is equivalent to 0.35% by weight, and a 20 millimolar

aqueous solution of K_2HPO_3 is equivalent to 0.32% by weight. Alternatively, the amount of the first salt is equal to one part by weight and the amount of the second salt is equal to between 0.001 and 1,000 parts by weight, i.e. the weight ratio of first salt to second salt is 1:0.001 to 1:1,000. It is preferred if the aqueous composition is comprised of 21.7% K_2HPO_4 and 21.5% K_2HPO_3 or 11.8% PO_4 and 10.7% PO_3, all of which are soluble.

Once formed, the composition will be applied to various plants to prevent fungus infection, particularly infection by the Phytophthora genus, and more particularly *Phytophthora infestans* infection. The preferable method of application is foliar, either by ground or aerial equipment, but is not limited to that method alone. Injection or soil applications, for example, could also be efficacious depending on specific crops and pathogens. While it is preferred to apply the composition in an aqueous solution, other forms of application may be used, including dusts, flowables, water dispersable granules, granules and inert emulsions, as well as oils. At least one application should be made; however, multiple applications of the composition can be made.

The inventive composition has utility on fruit crops, agronomic crops, ornamentals, trees, grasses, vegetables, grains, and floricultural crops, as well as some aquatic crops, including water cress. The crops most likely infected by *Phytophthora infestans* are potatoes (*Solanum tuberosum*) and tomatoes (*Lycopersicon esculentum*). As such, the present composition is especially useful in treating potato and tomato plants to prevent Phytophthora infection.

The following examples set forth the preferred concentrations and techniques for formulation thereof, as well as methods of application, use and test results demonstrating the efficacy of the inventive concentration in protecting plants against attack by *Phytophthora infestans*. It is to be understood, however, that these Examples are presented by way of illustration only, and nothing therein shall be taken as a limitation upon the overall scope of the invention. The specific components tested in the Examples were prepared and applied as follows.

In each of Examples 11 and 12, treatments were applied as a one gallon solution by a back pack sprayer, maintained at about 60 psi, in sufficient quantities of water to achieve thorough coverage. The spray rate used was equivalent to approximately 25

gallons per acre. All treatments were applied to the appropriate number of experimental units assigned in a randomized complete block (CRB) design replicated four times.

As used in the following examples, "Percent Late Blight" means the percent of plants that exhibit blight. "Lesions Per Plant" relate to the number of lesions on a particular plant caused by the infectious inoculum. The "No. Infected Leaflets" relates to the number of infected leaves per plant.

Example 11.

Potatoes (*Solanum tuberosum,* variation Atlantic) were infected with a pathogen, *Phytophthora infestans,* to determine whether suitable treatments could be developed to eliminate the pathogen from the infected plants and, more importantly, prevent infection of the plants by the pathogen. The Phytophthora pathogen causes late blight in infected plants. The plants were treated with the below listed compositions, twice, with the applications being seven (7) days apart. The composition of the inoculant added to the plants is listed below in the table. One week (7 days) after the last inoculation was made to the plants, the potato plants were then infected with the pathogen, *Phytophthora infestans*. The infectious inoculum was equal to 12,000 sporangia per millimeter (ml), with 20 ml administered per plant. The *Genotype* of the pathogen was US-8 and the Matingtype was A2. Seven days after inoculation with the pathogen, the results were tabulated to determine the percentage of blight in the plants and the number of lesions per plant. Additionally, the number of infected leaflets per plant were tabulated. The results are as follows:

SUMMARY LATE BLIGHT OBSERVATIONS
GREENHOUSE EXPERIMENT

TREATMENT	RATE/ACRE	% LATE BLIGHT	LESIONS PER PLANT	NO. INFECTED LEAFLETS PER PLANT
K_2HPO_3 + K_2HPO_4	1 % v/v + 1 % v/v	0.39	0.5	0.5
Cu-EDDHA	0.2 lb. AI	12.30	35.3	26.9
K_2HPO_3	1 % v/v	1.85	2.4	1.8
K_2HPO_4	1 % v/v	18.45	41.4	31.1
CONTROL		28.12	84.4	50.1

Tests were made on single 6" pots x 4 reps in CRB design.

As can be seen, an inoculum of just phosphonate (PO_3) showed good results in controlling the blight. However, better results were achieved using the phosphate (PO_4) and phosphonate (PO_3) composition. The (PO_4) and (PO_3) combination demonstrated exceptional blight depression, indicating that potato blight can be better controlled using a composition comprised of (PO_3) and (PO_4). This indicates that a synergistic effect is achieved with a (PO_3) and (PO_4) combination.

Example 12.

Tomatoes (*Lycopersicon esculentum*, FL 40) were infected with a pathogen, *Phytophthora infestans,* to determine whether suitable treatments could be developed to prevent infection of the plants by the pathogen. The Phytophthora pathogen causes late blight in infected plants. The plants were treated with the below listed compositions, twice, with the application dates being seven (7) days apart. The composition of the inoculant added to the plants is listed below in the table. One week (7 days) after last inoculation was made to the plants, the tomato plants were then infected with the pathogen, *Phytophthora infestans*. The infectious inoculum was equal to 12,000 sporangia per millimeter (ml), with 20 ml administered per plant. The *Genotype* of the pathogen was US-17 and the Matingtype was A1. Seven days after inoculation with the pathogen, the results were tabulated to determine the percentage of blight in the plants

and the number of lesions per plant. Additionally, the number of infected leaflets per plant were tabulated. The results are as follows:

GREENHOUSE TOMATO LATE BLIGHT TRIAL

TREATMENT	RATE/ACRE	LESIONS PER PLANT	NO. INFECTED LEAFLETS PER PLANT
K_2HPO_3 + K_2HPO_4	2% v/v	6.0	2.5
SIMAZINE 4L	0.1 lb. AI	52.3	36.8
K_2HPO_3	1% v/v	56.7	21.5
K_2HPO_4	1% v/v	74.8	36.5
CONTROL		66.8	33.8

Excellent results were achieved using the phosphate (PO_4) and phosphonate (PO_3) composition. The (PO_4) and (PO_3) combination demonstrated exceptional blight depression, indicating that the blight can be better controlled using a composition comprised of (PO_3) and (PO_4). This indicates that a synergistic effect is achieved with a (PO_3) and (PO_4) combination.

The above Examples demonstrate that the inventive compositions are useful in protecting plants against attack by the *Phytophthora infestans* infection with the application of one solution.

The disclosures in all references cited herein are incorporated by reference.

Alternatively, the composition of the invention can be used to prevent infection by *Phycomycetes*, *Ascomycetes*, and other fungal pathogens, as well as bacteria.

In the following Examples, the composition of the samples tested are as follows:

Sample 1: 15.1% K_2HPO_3 + 15.1% K_2HPO_4 + 69.8% inert ingredients (H_2O)

Sample 2: Cu EDDHA (3.2% Cu) - 0.2 lb AI/100 gal.

Sample 3: 80% v/v Sample 1 + 1 lb. 6% Fe-EDDHA

Sample 4: 80% v/v Sample 1 + 20% v/v 6% Mn-EDTA

Sample 5: 80% v/v Sample 1 + 20% v/v 10% Zn-EDTA

Sample 6: 80% v/v Sample 1 + 10% v/v 6% Mn-EDTA + 10% v/v 10% Zn-EDTA

Sample 7: 80% v/v Sample 1 + 0.2 lb. AI/100 gal. (Cu-EDDHA)

Example 13.

Single 5 gal. containers of live oak trees (*Quercus virginia*), inoculated with *Phyllactinia guttata*, were treated using a SOLO backpack sprayer at 60 psi at 25 gpa using the following compositions. Treatments were replicated four times and assigned in a randomized complete block design.

LIVE OAK POWDERY MILDEW TRIAL

TREATMENT	RATE/100 GALLONS (V/V)	DISEASE SEVERITY PRE-APPLICATION 4/20	POST-APPLICATION 6/15	PHYTO-TOXICITY
1) Sample 1	2 gal.	3.0	2.0	0
2) Sample 2	0.2 lb. AI	2.75	2.5	0
3) Sample 3	2 gal.	2.5	2.0	0
4) Sample 4	2 gal.	3.0	1.5	0
5) Sample 7	2 gal.	3.0	1.0	0
6) CONTROL	-	3.0	4.0	0

Powdery Mildew - *Phyllactinia guttata*

Appl. Dates: 4/27, 5/11, 6/1, and 6/15/00

Rated 6/15/00, Clay County, FL.

Disease Severity 1-5: 1=1-10%, 2=11-25%, 3=26-50%, 4=51-75% and 5=76-100%

PHYTOTOXICITY RATINGS: 0-10 0=no phytotoxicity, 10=100% kill

Example 14.

Single 5 gal. containers of live dogwoods (*Cornus florida*), inoculated with *Oidium spp.*, were treated using a SOLO backpack sprayer at 60 psi at 25 gpa using the following compositions. Treatments were replicated four times and assigned in a randomized complete block design.

DOGWOOD POWDERY MILDEW TRIAL

TREATMENT	RATE/100 GALLONS (V/V)	DISEASE SEVERITY PRE-EXISTING 4/27	DISEASE SEVERITY POST-APLICATION 6/15	PHYTO-TOXICITY
1) Sample 1	2 gal.	2.5	1.5	0
2) Sample 4	2 gal.	3.0	1.0	0
3) Sample 5	2 gal.	2.5	1.5	0
4) Sample 6	2 gal.	2.0	1.75	0
5) Sample 7	2 gal.	2.5	1.0	0
6) CONTROL	-	2.75	3.0	0

Powdery Mildew - *Oidium spp.*

Appl. Dates: 4/27, 5/11, 5/25, and 6/8/00

Rated 6/15/00

Disease Severity 1-5: 1=1-10%, 2=11-25%, 3=26-50%, 4=51-75% and 5=76-100%

PHYTOTOXICITY RATINGS: 0-10 0=no phytotoxicity, 10=100% kill

In Example 13 above, formulation Sample 7 demonstrates that the pre-application Disease Severity factor as measured on April 20, 2000, of 3.0 was reduced to 1.0 demonstrating the combination phosphate, phosphonates and Cu EDDHA (a metal chelate) is effective in treating the live oak powdery mildew. Similarly, Example 14 demonstrates that formulations Sample 4 (PO_3 + PO_4 + Mn EDTA) and Sample 7 (PO_3 + PO_4 + Cu EDDHA) are effective in treating dogwood powdery mildew as evidenced in disease severity from 3.0 to 1.0 and 2.5 to 1.0, respectively.

Thus, there has been shown and described a method of use for fungicidal and bactericidal compositions, which provide improved efficacy in controlling fungi and bacterial infections in plants. More particularly, the compositions and method related to metal chelates, and preferably a copper chelate in the form of Cu-EDDHA (disodium cupric ethylenediamine-di-o-hydroxyphenylacetic acid), in an aqueous solution, also including an effective amount of phosphate (PO_4) and phosphanate (PO_3), which fulfills all the objects and advantages sought therefore. It is apparent to those skilled in the art, however, that many changes, variations, modifications, and other uses and applications for the subject fungicidal and bactericidal compositions are possible and,

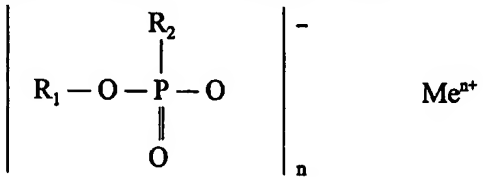
also, such changes, variations, modifications, and other uses and applications which do not depart from the spirit and scope of the invention are deemed to be covered by the invention which is limited only by the claims which follow.

CLAIMS

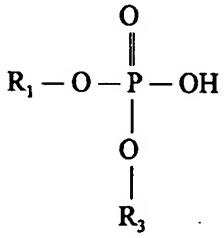
What is claimed is:

1. A composition for preventing and controlling fungicidal and bacterial diseases in plants, said composition comprising effective amounts of:

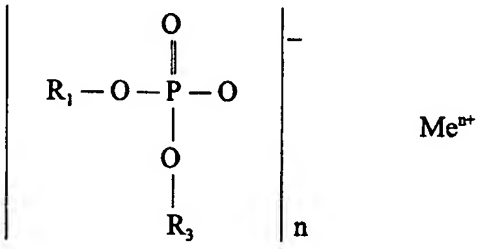
(a) at least one first salt having the following formula:



(b) at least one second salt selected from compounds having the formula:



or the formula:



where R_1 is selected from the group consisting of H, K, an alkyl radical containing from 1 to 4 carbon atoms, halogen-substituted alkyl or nitro-substituted alkyl radical, an alkenyl, halogen-substituted alkenyl, alkynl, halogen-substituted alkynl, alkoxy-substituted alkyl radical, ammonium substituted by alkyl or hydroxy alkyl radicals;

R_2 and R_3 are selected from the group consisting of H and K;

Me is selected from the group consisting of K, alkaline earth metal cations, an aluminum atom, and an ammonium cation;

n is a whole number equal to between 1 and 3, equal to the valence of Me; and

(c) at least one metal chelate wherein said metal is a metal selected from rows 4 or 5 of the periodic table of the elements.

2. The composition of Claim 1 wherein said metal chelate is present in said aqueous solution in amount equal to from about 0.01 to about 2 pounds AI per acre.

3. The composition of Claim 2 wherein said metal chelate is present in said aqueous solution in amount equal to from about 0.01 to about 0.8 pounds AI per acre.

4. The composition of Claim 1 wherein said metal is a metal selected from row 4 of the periodic table of the elements.

5. The composition of Claim 1 wherein said metal chelate has a solubility equal to about 100% where at least 80 pounds of said metal chelate are dissolved in 100 gallons of water at 50° C.

6. The composition of Claim 1 wherein said metal chelate is added as an aqueous solution containing an amount of metal chelate (on a metal basis) equal to between 1% and 5% by weight of the aqueous solution.

7. The composition of Claim 1 wherein said metal constituent is selected from the group consisting of iron, zinc, tin, manganese, copper, and combinations thereof.

8. The composition of Claim 7 wherein said metal constituent is selected from the group consisting of zinc, manganese, copper, and combinations thereof.

9. The composition of Claim 8 wherein said metal chelate is selected from the group consisting of Cu-EDDHA, Cu-pEDDHA, Cu-EDDHMA, and combinations thereof.

10. The composition of Claim 1 wherein said chelate constituent is selected from the group consisting of pEDDHA, EDDHA, and EDDHMA.

11. The composition of Claim 1 wherein said first salt is selected from the group consisting of K_2HPO_3, KH_2PO_3, $(NH_3)H_2PO_3$, and $(NH_3)_2HPO_3$; and said second salt is selected from the group consisting of K_2HPO_4, KH_2PO_4, K_3PO_4,

$(NH_3)_2HPO_4$, $(NH_3)H_2PO_4$, and $(NH_3)_3PO_4$.

12. The composition of Claim 1 wherein said composition is in an aqueous solution, wherein each said first and second salt is present in solution from about 0.1 millimolar to about 1000 millimolar.

13. The composition of Claim 12 wherein said composition is in an aqueous solution, wherein each said first and second salt is present in solution from about 20 millimolar to about 200 millimolar.

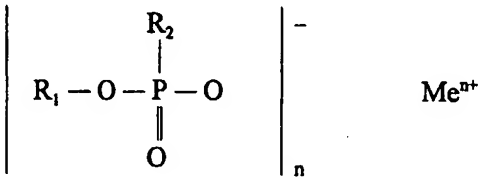
14. The composition of Claim 1 wherein the weight ratio of said first salt to said second salt is 1:0.001 to 1:1,000.

15. The composition of Claim 1 wherein said composition treats or prevents diseases caused by Phytophthora.

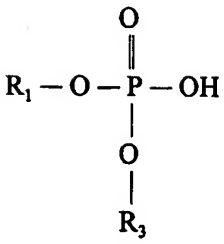
16. The composition of Claim 15 wherein said composition treats or prevents diseases caused by *Phytophthora infestans*.

17. The composition of Claim 1 wherein the plants are tomato and potato species.

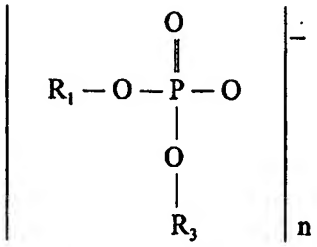
18. A method for controlling fungicidal and/or bacterial disease wherein said method comprises applying to a plant fungicidally and/or bactericidally effective amounts of at least one metal chelate, at least one phosphate salt, and at least one phosphonate salt in aqueous solution, wherein said phosphonate salt has the formula:



said phosphate salt is selected from compounds having the formula:



or the formula:



where R_1 is selected from the group consisting of H, K, an alkyl radical containing from 1 to 4 carbon atoms, halogen-substituted alkyl or nitro-substituted alkyl radical, an alkenyl, halogen-substituted alkenyl, alkynl, halogen-substituted alkynl, alkoxy-substituted alkyl radical, ammonium substituted by alkyl or hydroxy alkyl radicals;

R_2 and R_3 are selected from the group consisting of H and K;

Me is selected from the group consisting of K, alkaline earth metal cations, an aluminum atom, and an ammonium cation;

n is a whole number equal to between 1 and 3, equal to the valence of Me; and said metal is a metal selected from rows 4 or 5 of the periodic table of the elements.

19. The method of Claim 18 wherein said metal chelate is present in said aqueous solution in amount equal to from about 0.01 to about 2 pounds AI per acre.

20. The method of Claim 19 wherein said metal chelate is present in said aqueous solution in amount equal to from about 0.01 to about 0.8 pounds AI per acre.

21. The method of Claim 18 wherein said metal is a metal selected from row 4 of the periodic table of the elements.

22. The method of Claim 18 wherein said metal chelate has a solubility equal to about 100% where at least 80 pounds of said metal chelate are dissolved in 100 gallons of water at 50° C.

23. The method of Claim 18 wherein said metal chelate is added as an aqueous solution containing an amount of metal chelate (on a metal basis) equal to between 1% and 5% by weight of the aqueous solution.

24. The method of Claim 18 wherein said metal constituent is selected from the group consisting of iron, zinc, tin, manganese, copper, and combinations thereof.

25. The method of Claim 24 wherein said metal constituent is selected from the group consisting of zinc, manganese, copper, and combinations thereof.

26. The method of Claim 25 wherein said metal chelate is selected from the group consisting of Cu-EDDHA, Cu-pEDDHA, Cu-EDDHMA, and combinations thereof.

27. The method of Claim 18 wherein said chelate constituent is selected from the group consisting of pEDDHA, EDDHA, and EDDHMA.

28. The method of Claim 18 wherein said first salt is selected from the group consisting of K_2HPO_3, KH_2PO_3, $(NH_3)H_2PO_3$, and $(NH_3)_2HPO_3$; and said second salt is selected from the group consisting of K_2HPO_4, KH_2PO_4, K_3PO_4, $(NH_3)_2HPO_4$, $(NH_3)H_2PO_4$, and $(NH_3)_3PO_4$.

29. The method of Claim 18 wherein said composition is in an aqueous solution, wherein each said first and second salt is present in solution from about 0.1 millimolar to about 1000 millimolar.

30. The method of Claim 30 wherein said composition is in an aqueous solution, wherein each said first and second salt is present in solution from about 20 millimolar to about 200 millimolar.

31. The method of claim 18 wherein the weight ratio of said first salt to said second salt is 1:0.001 to 1:1,000.

32. The method of claim 18 wherein said composition treats or prevents diseases caused by Phytophthora.

33. The method of claim 32 wherein said composition treats or prevents diseases caused by *Phytophthora infestans*.

34. The method of claim 18 wherein the plants are tomato and potato species.

35. A method of controlling fungicidal and/or bactericidal disease in plants comprising applying to the plants in enhanced fungicidally and/or bactericidally effective amounts an aqueous composition comprising:

(a) an aqueous solution of H_3PO_3 and KOH,

(b) an aqueous solution of monopotassium phosphate and KOH, and

(c) a metal chelate wherein said metal is a metal selected from rows 4 or 5 of the periodic table of the elements.

36. The method of Claim 35 wherein the amount of potassium phosphonate in said aqueous solution (a) and the amount of potassium phosphate in said aqueous solution (b) is each present in said composition in an amount from about 0.1 millimolar to about 1000 millimolar

37. The method of Claim 35 wherein the weight ratio of potassium phosphonate prepared from solution (a) in said composition to potassium phosphate prepared from solution (b) in said composition is 1:0.001 to 1:1,000.

38. The method of Claim 35 wherein said metal chelate is present in said aqueous solution in amount such that the metal is applied to the plants at a rate of from about 0.01 to about 2 pounds AI per acre.

39. The method of Claim 35 wherein said chelate constituent is selected from pEDDHA, EDDHA, EDDHMA or combinations thereof, and said metal is selected from iron, zinc, tin, manganese, copper, and combinations thereof.

40. A method of controlling fungicidal and/or bactericidal disease in plants comprising applying to the plants in enhanced fungicidally and/or bactericidally effective amounts an aqueous composition prepared by mixing:

(a) an aqueous solution of H_3PO_3 and KOH,

(b) an aqueous solution of monopotassium phosphate and KOH, and

(c) a metal chelate wherein said metal is a metal selected from rows 4 or 5 of the periodic table of the elements.

41. The method of Claim 40 wherein the amount of potassium phosphonate in said aqueous solution (a) and the amount of potassium phosphate in said aqueous solution (b) is each present in said composition in an amount from about 0.1 millimolar to about 1000 millimolar

42. The method of Claim 40 wherein the weight ratio of potassium phosphonate prepared from solution (a) in said composition to potassium phosphate prepared from solution (b) in said composition is 1:0.001 to 1:1,000.

43. The method of Claim 40 wherein said metal chelate is present in said aqueous solution in amount such that the metal is applied to the plants at a rate of from about 0.01 to about 2 pounds AI per acre.

44. The method of Claim 40 wherein said chelate constituent is selected from pEDDHA, EDDHA, EDDHMA or combinations thereof, and said metal is selected from iron, zinc, tin, manganese, copper, and combinations thereof.

45. A method of controlling fungicidal and/or bactericidal disease in plants comprising applying to the plants in enhanced fungicidally and/or bactericidally effective amounts an aqueous composition comprising:

(a) an aqueous solution of H_3PO_3 and KOH,

(b) an aqueous solution of dipotassium phosphate, and

(c) a metal chelate wherein said metal is a metal selected from rows 4 or 5 of the periodic table of the elements.

46. The method of Claim 45 wherein the amount of potassium phosphonate in said aqueous solution (a) and the amount of potassium phosphate in said aqueous solution (b) is each present in said composition in an amount from about 0.1 millimolar to about 1000 millimolar

47. The method of Claim 45 wherein the weight ratio of potassium phosphonate prepared from solution (a) in said composition to potassium phosphate prepared from solution (b) in said composition is 1:0.001 to 1:1,000.

48. The method of Claim 45 wherein said metal chelate is present in said aqueous solution in amount such that the metal is applied to the plants at a rate of from about 0.01 to about 2 pounds AI per acre.

49. The method of Claim 45 wherein said chelate constituent is selected from pEDDHA, EDDHA, EDDHMA or combinations thereof, and said metal is selected from iron, zinc, tin, manganese, copper, and combinations thereof.

50. A method of controlling fungicidal and/or bactericidal disease in plants comprising applying to the plants in enhanced fungicidally and/or bactericidally effective amounts an aqueous composition prepared by mixing:

(a) an aqueous solution of H_3PO_3 and KOH,

(b) an aqueous solution of dipotassium phosphate, and

(c) a metal chelate wherein said metal is a metal selected from rows 4 or 5 of the periodic table of the elements.

51. The method of Claim 50 wherein the amount of potassium phosphonate in said aqueous solution (a) and the amount of potassium phosphate in said aqueous solution (b) is each present in said composition in an amount from about 0.1 millimolar to about 1000 millimolar

52. The method of Claim 50 wherein the weight ratio of potassium phosphonate prepared from solution (a) in said composition to potassium phosphate prepared from solution (b) in said composition is 1:0.001 to 1:1,000.

53. The method of Claim 50 wherein said metal chelate is present in said aqueous solution in amount such that the metal is applied to the plants at a rate of from about 0.01 to about 2 pounds AI per acre.

54. The method of Claim 50 wherein said chelate constituent is selected from pEDDHA, EDDHA, EDDHMA or combinations thereof, and said metal is selected from iron, zinc, tin, manganese, copper, and combinations thereof.